High Pressure Liquid Chromatographic Quantification of Nitrile Biocatalysis

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Abstract

Nitrile biocatalysts are of use in the chemical and pharmaceutical industries for the synthesis of carboxyamides and carboxylic acids. In particular, the application of biocatalysts in the synthesis of single enantiomer compounds is of increasing interest, but requires novel substrate specific highly stereoselective biocatalysts. Addition to the limited toolbox of known nitrile biocatalysts requires definitive characterisation of the biocatalysts through accurate determination of the substrate profiles and quantification of activity. The accurate quantification of stereoisomers chiral mixtures to determine biocatalyst stereoselectivity remains a significant challenge due to the difficulty in separating stereoisomers by physical methods.

The known nitrile metabolising organism, *Rhodococcus rhodochrous* ATCC BAA-870, was grown in a defined medium and harvested, providing whole cell biocatalyst. Additional biomass was disrupted to provide a cell free enzyme extract, which was put through an enzyme purification protocol to provide a solution with specific activity of 351 $U.mg^{-1}$. A portion of the enzyme was self immobilised using the SphereZyme™ technique. The nitrile hydratase SphereZymes™ (1.2 $U.mg^{-1}$ initial activity) that were prepared had pH and temperature optima of 6 and 30°C respectively, and could be recovered by repeated washing. The particles retained activity in the presence of the organic solvents isooctane and n-hexadecane saturated with 50 mM phosphate buffer (pH 7.5).

An initial analytical system was devised for quantification of the nitrile hydratase activity using the non-chiral substrate benzonitrile. An improved reversed phase high performance liquid chromatography method was developed to separate and quantify benzamide, benzoic acid and benzonitrile. The mobile phase consisting of 0.1% trifluoroacetic acid in H_2O and acetonitrile (70:30, %v/v), at a flow rate of 0.5 $ml.ml^{-1}$, 25°C, resolved all three analytes in 3.5 minutes on a Waters X-Terra MS C18 3.5µm column. UV detection was carried out at 210 nm.

Analytical methods to determine activity and enantioselectivity of the whole cell biocatalyst were subsequently developed for both β-amino nitriles and β-hydroxy nitrile substrates and hydrolysis products.

List of Outputs

Publications arising from this study

Kinfe H.H., Chhiba V., Frederick J., Bode M.L., **Mathiba K.**, Steenkamp P.A. and Brady D. (**2009**). Enantioselective hydrolysis of β-hydroxy nitriles using the whole cell biocatalyst of *Rhodococcus rhodochrous* ATCC BAA-870. J. Mol. Cat. B: Enz., **59**: 231-236.

Chhiba V.P., Bode M., **Mathiba K.**, Kwezi W., and Brady D. (**2012**). Enantiomeric biocatalytic hydrolysis of β-aminonitriles to β-aminoamides using *Rhodococcus rhodochrous* ATCC BAA-870. J Mol Cat B: Enz., **76**: 68– 74.

Both papers are direct results of the research contain herein, particularly chapter 4. My role was the development of non-chiral and chiral methods for quantification of compounds present in various β-substituted nitriles biocatalysis reactions. I analysed the analytical data independently, and provided novel insight into the meaning of data. I wrote the analytical sections of both publications. I assisted with the synthesis and biocatalysis of the nitrile substrates. Dr Steenkamp assisted with structure analysis and confirmation of all new and unknown compounds. Dr Kinfe, Dr Bode, Ms Chhiba, and Ms Kwezi assisted with designing and performing synthesis and biocatalysis of nitriles. Ms Frederick assisted with supplying the biocatalyst. Dr Brady supervised the project.

Scientific Magazines Article

Mathiba, K., Molawa, L., Jordaan, J., Moolman, S., Gardiner, D. and Brady, D. (**2009**). Novel Immobilised Biocatalysis. Chemical Technology, June 10-12.

This article is a direct result of literature research (Chapter 1) contained herein, particularly immobilisation sub-section. I was responsible for writing the article. Ms

Molawa, Dr Jordaan, Dr Moolman, and Dr Gardiner assisted with review of the article. Dr Brady assisted with structuring and editing the final version of the article.

Posters and Conference Proceedings

Kinfe H.H., Chhiba V.P., Frederick J., **Mathiba K.**, and Brady D (**2008**). Application of stereoselective biocatalysts for the enantiomeric resolution of beta-hydroxynitriles. Published in J. Biotechnology, **136**, Supplement 1, October 2008.

Gulston L., Chhiba V., Visser D., **Mathiba K**., Brady D. and Roux van der Merwe R. (**2009**). Biodegradation of Nitriles Using Soil Isolates. The Biennial National Conference of the South African Society for Microbiology, Durban, South Africa from 20-23 September 2009 (Poster).

Chhiba V.P., Frederick J., Kinfe H., Gulston L., Kwezi W., **Mathiba K.**, Visser D., M. L. Bode and D. Brady (**2010**). Biocatalytic Hydrolysis of β-Substituted Nitriles and Dinitriles. 5th International Congress on Biocatalysis, Hamburg, Germany. August 29 - September 2, 2010 (Oral).ISBN 978-3-941492-24-0.

Chhiba V. P., Frederick J., Kinfe H., Gulston L., Kwezi W., **Mathiba K**., Visser D., Bode M. L. and Brady D. (**2010**) Biocatalytic Hydrolysis of β-Substituted Nitriles and Dinitriles. COST Action CM0701, Vilnius, Lithuania. 8 - 11 September, 2010. (Oral).

I analysed the data independently and provided insight into the meaning of all the results presented in the above presentations.

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List of Abbreviations

3H4PPN	3-hydroxy-3-phenylpropanenitrile
3OH4PBN)	3-Hydroxy-4-phenoxybuanenitrile
4Bz3HBN	4-(benzyloxy)-3-hydroxybutanenitrile
4(4ChlP) 3HBN	4(4-chlorophenyl)-3-hydroxybutanenitrile
A	Absorbance
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
AGP	Acid glycoprotein
ATCC	American Type Culture Collection
BAA-870	Rhodococcus rhodochrous ATCC BAA-870
BSA	Bovine serum albumin
°C	Degree Centigrade
C2	Carbon at position number 2
C-3 or C3	Carbon at position number 3
C-6	Carbon at position number 6
$Ca.Cl_2.2H_2O$	Calcium chloride dihydrate
CBH	Cellobiohydrolyase
CD	Cyclodextrin
CLE	Cross-linked enzymes
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
cm	Centimeter
CN	Cyano group
^{13}C-NMR	Carbon-13 Nuclear magnetic resonance
Co(III)	Cobalt three ion
CSDE	Cross-linked spray dried enzymes
CSP	Chiral stationary phase
Cys	Cystine
Cys-SOH	Cystine- sulfinic acid

DCM	Dichloromethane
K_2HPO_4	Dipotassium hydrogen phosphate
EDA	Ethylenediamine
ee	Enantiomeric excess
EtOAC	Ethyl acetate
EtOH	Ethanol
Fe(III)	Iron three ion
$FeSO_4.7H_2O$	Iron sulphate heptahydrate
FTIR	Fourier transform Infra Red
g	Grams
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
$g.l^{-1}$	Grams per litre
$g.l^{-1}.h^{-1}$	Grams per litre per hour
Glu	Glutamic acid
Glut	Glutaraldehyde
h	Hour
$HClO_4$	Perchloric acid
Hex	Hexane
^1H-NMR	Proton Nuclear magnetic resonance
HPLC	high performance liquid chromatography
HPLC-MS	HPLC- Mass spectrometry
HSA	Human serum albumin
i.d.	Internal diameter
IPA	Isopropanol or Isopropan-2-ol
kDa	Kilodaltons
$Kg.l^{-1}$	Kilogram per litre
KH_2PO_4	Potassium dihydrogen phosphate
KPa	Kilo Pascal
L	length
kPsi	Kilopound-force per square inch

l	Litre
LCMS	Liquid Chromatography Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of Quantification
Lys	Lysine
M	Molar
MeOH	Methanol
Met	Methionine
$MgSO_4$	Magnesium sulphate
mM	Millimolar
mg	Milligram
min	Minute
ml	Millilitre
% m/m	Percent mass per mass
MS	Mass spectrometry
% m/v	Percent mass per volume
NaCl	Sodium Chloride
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2CO_3	Sodium Carbonate
NH	Amine group
Nhase	Nitrile hydratase
NHSZ	Nitrile hydratase Spherezymes™
Nit	Nitrilase
NitFhit	Nitrilase-fragile histidine triad fusion protein
OD	Optical density
OH	Hydroxyl group
PDA	Photodiode array
PDMS	Poly (dimethylsilicoxane)
PHEA	Poly (2-hydroxyethyl acrylate)
PEI	Polyethyleneimine
psi	Pound-force per square inch

R	Ristocetin
R^2	Correlation coefficient
RP	Reversed phased
RP-HPLC	Reversed-phase high performance liquid chromatography
rpm	Revolutions per minute
RSD	Relative standard deviation
R_t	Retention time
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SZ	Spherezymes™
T	Teicoplanin
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TMS	Tetramethylsilane
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Tris-HCl	Tris-hydrochloride
TSB	Tryptone soya broth
µl	Microlitre
V_{max}	Maximum velocity at saturating conditions of the substrate
% v/v	Percent volume per volume
$U.mg^{-1}$	Units per milligram
UV	Ultraviolet

The IUPAC-IUBMB three letter codes are used for amino acids.

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Chapter 1: Literature Review

1.1. Introduction

Enzymes are useful catalysts that are well-equipped to utilise a wide range of complex molecules as substrates. Moreover, many enzymes are highly substrate selective and are capable of catalysing reactions with high chiral and positional or regional selectivities (Schmid *et al.*, 2001). Chiral biocatalysis offers a further advantage since the tedious protection and de-protection steps required in many enantio- and regioselective organic syntheses are negated with the use of enzymes (Schmid *et al.*, 2001). Many traditional organic synthetic routes also have problems associated with unwanted by products (due in some cases to low stereo- or regio-selectivity), low yields, and high consumption of energy. Enzyme based reactions are often more specific, exhibiting fewer side reactions and therefore limit unwanted side products. This atom efficiency and specificity are the basis for the contribution of biocatalysts in green chemistry.

Biocatalysts do suffer from some general drawbacks, however, as enzymes may display limited duration in use or short shelf lives due to instability towards temperature, pH, solvents, storage and mechanical shear (Cao, 2005a; Mateo *et al.*, 2007). This is a commercial concern as enzymes are often still relatively expensive, and this result in high running costs in industry. Therefore most enzymes have to be stabilised before industrial application. Since 1950, numerous efforts have been devoted to improvement of industrial enzymes by immobilisation (Kilara and Shahani, 1979; Bornscheuer, 2003; Cao, 2005a). This was associated with efforts of making enzymes more rigid, by immobilisation “entrapment” methods intended to retain major catalytic functions or properties of the native enzymes, and increasing the overall size of the biocatalyst. Although the main goal of immobilisation is often extended activity, it also provides for easy recovery of the enzyme from the reaction media or product (Cao *et al.*, 2003) permitting catalyst recycling.

1.2. Nitrile Hydrolysing Enzymes

Nitriles are used in industrial organic synthesis as precursors to carboxylic amides and acids, for example in the production of bulk polymers as well as in the synthesis of antibiotics. For example, adiponitrile is produced as a precursor of acrylic fibers and plastics (Zhou *et al.*, 2005). Borrelidin, a nitrile containing macrolide antibiotic produced from the *Streptomycin parvulus* possesses anticancer, antiangiogenesis, antivirial and antimalarial activity (Vino and Lokesh, 2008). Applications of nitrile converting enzymes provide a much more efficient method than the traditional synthetic routes, which involve high temperatures and extremes of pH. Biocatalysts operate under mild conditions of temperature, pressure, pH, which save energy and do not produce undesirable by-products. For example, a route via enzymatic hydrolysis of β-amino nitrile does not involve the production of any waste (Van Rantwijk, 2000).

Nitrile hydrolysis occurs by two distinct mechanisms which include either a single step process catalysed by nitrilase (EC 3.5.5.1), or a nitrile hydratase (EC 4.2.1.84) and amidase (EC 3.5.1.4) catalysed sequential reaction (figure 1.1). In general, aromatic nitriles are metabolised to the corresponding acid in a single-step reaction by nitrilases. Aliphatic nitriles, on the other hand, are converted to amides by nitrile hydratases and then hydrolysed by amidases to carboxylic acids. Exceptions to this include the nitrile hydratases of a *Rhodococcus* sp. (Hjort *et al*., 1990), *Bacillus smithii* SCJ05-1 (Takashima,1995), and the nitrilase of *Rhodococcus rhodochrous* K22 (Kobayashi *et al.,* 1990), which are capable of hydrolysing both aliphatic and aromatic nitriles.

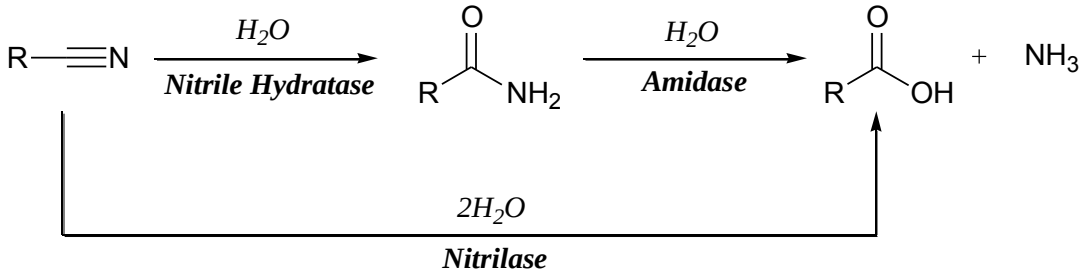


Figure 1.1: Nitrile hydrolysis catalysed by distinct pathways.

1.2.1. Nitrilase (Nit)

Nitrilase (EC 3.5.5.1) catalyses the hydrolysis of nitriles to the corresponding acids and ammonia by adding two molecules of water to the nitrile in a single step without the intermediate formation of the corresponding amide.

Distribution: Nitrilases are widely expressed in prokaryotes and eukaryotes. These enzymes belong to a superfamily which includes amidases and carbamylases, all having in common the Glu, Lys, Cys catalytic triad responsible for activity in all enzymes in the family (Banerjee *et al.*, 2002; Brenner, 2002; Piotrowski, 2008). They have wide applications in industry, for instance, in the removable of toxic compounds such as acrylonitrile, acetonitrile, propanenitrile and butanenitrile from waste water or spillage (Martínková *et al*., 2008).

Enzyme Structure and homology: There are four atomic structures of distinct homologs in the superfamily, namely 1ems, 1erz, 1f89 and 1j31 (Pace *et al.*, 2000). All these structures have two-fold symmetry which conserves the αßßα-αßßα fold (figure 1.2) across the dimer interface as the A surface (Thuku *et al.*, 2009). Some of these homologs exist as homo-oligomers. The nitrilase J1 is reported to oligomerise and acquire activity in the presence of benzonitrile. A nitrilase isolated from *Rhodococcus rhodochrous* ATCC 39484 is reportedly converted to an active form by subunit association when incubated with substrate (Stevenson *et al.*, 1992) or when in the presence of higher concentrations of enzyme, salt or organic solvent (Nagasawa *et al.*, 2000). These conditions may result in a hydrophobic effect that changes the

conformation of the enzyme in such a way so as to expose hydrophobic sites which enable subunit assembly and hence enzyme activation.

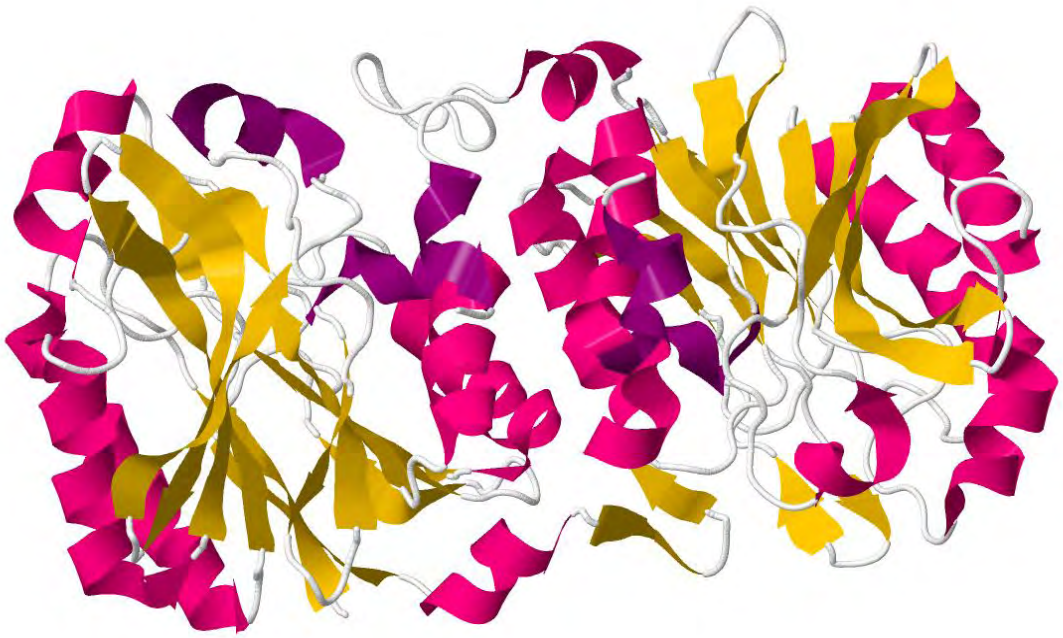


Figure 1.2: Crystallographic structure of *Pyrococcus abyssi* nitrilase.
(reproduced from Raczynska *et al.*, 2011).

Substrate Specificity: Nitrilases are classified into three different categories based on their substrate specificity. These are: (i) aromatic nitrilases which hydrolyse aromatic or heterocylic nitriles to corresponding acids and ammonia; (ii) aliphatic nitrilases which hydrolyse aliphatic nitriles or arylacetonitriles to their respective carboxylic acids and cyanide dehydrates and (iii) arylacetonitrilases which act on arylacetonitriles. The best characterised nitrilase is that from *Rhodococcus rhodochrous* J1, which was found to be specific for aromatic or heterocyclic nitriles such as benzonitrile, 3-chlorobenzonitrile and 2-thiophenecarbonitrile (Kobayashi *et al*., 1989, Thuku *et al.*, 2009). The nitrilase from *Rhodococcus* ATCC 39484 was reported to prefer hydrophobic aromatic substrates (Stevenson *et al.*, 1992). The nitrilase from *Acidovirax facilis* was shown to be efficient at hydrolysing aliphatic dinitriles to cyanoacid intermediates and had

preference for C-3 –C-6 dinitriles over mononitriles of the same chain length (Gavagan *et al.*, 1999).

Reaction Mechanism: The nitrilase reaction mechanism (figure 1.3) probably involves a nucleophillic attack on the nitrile carbon by a thiol group of Cys residue, forming a covalently bound enzyme substrate complex, thiomidate intermediate. The hydrolysis steps that follows involves an attack by two water molecules and protonation of the nitrogen atom, which is removed as ammonia (Banerjee *et al.*, 2002; Piotrowski,2008; Thuku *et al.*, 2009).

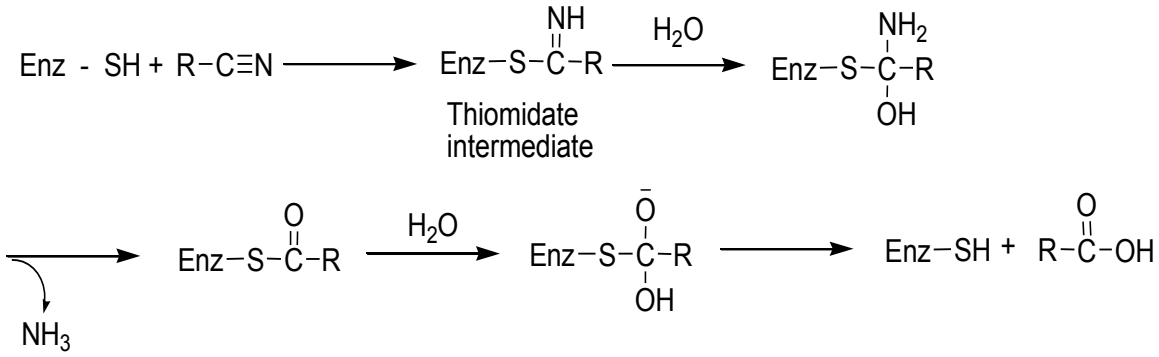


Figure 1.3: The reaction mechanism of nitrilases
(after Banerjee *et al.*, 2002).

Commercial Applications: Nitrilases have a wide range of industrial applications, for instance in the manufacture of nicotinic acid, ibuprophen, acrylic acid and detoxification of cynanide waste (Pace *et al.*, 2000; Banerjee *et al.*, 2002). Nitrilases are used as biocatalyst because of their specificity and enantioselectivity (Thuku *et al.*, 2009). For example, some of the nitrilases discovered by Robertson *et al*. (2004) from various environmental sources were reported to be able to hydrolyse mandelonitrile, hydroxyglutaronitrile, and phenylacetaldehyde cyanohydrins to corresponding acids with enantiomeric excess (ee) above 90% for the preferred enantiomer.

1.2.2. Nitrile Hydratase (Nhase)

Nitrile hydratase (EC 4.2.1.84) is an enzyme that converts nitrile groups to the corresponding amides by addition of a water molecule (figure 1.4), which may then be converted by an amidase to the corresponding carboxylic acid plus ammonia.

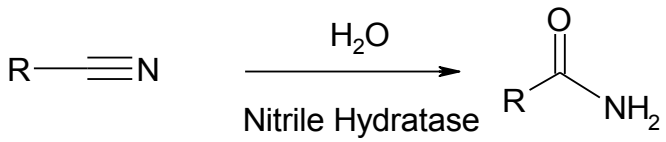


Figure 1.4:Nitrile biocatalysis by nitrile hydratase.

The enzyme structure: Nitrile hydratase is a α, β-heterodimeric metalloenzyme (figure 1.5) composed of subunits ranging in size from 26 to 35 kDa (Banerjee *et al.,* 2002). Nitrile hydratases are classified according to their catalytically essential metal cation in the α -subunit in the active site that is a catalyst for -CN hydration, with the ferric nitrile hydratases containing a non-haem Fe(III), and the cobalt nitrile hydratases containing a non-corrinoid Co(III). Fe(III)- and Co(III)-type Nhase enzymes differ in biotransformation activity and substrate specifity, although their amino acid sequences exhibit significant homology. The iron containing nitrile hydratase active site is interesting in that it is light activated at the non-haem iron centre on the β-subunit, and activation occurs by displacement of nitric oxide bound to the non-haem iron.

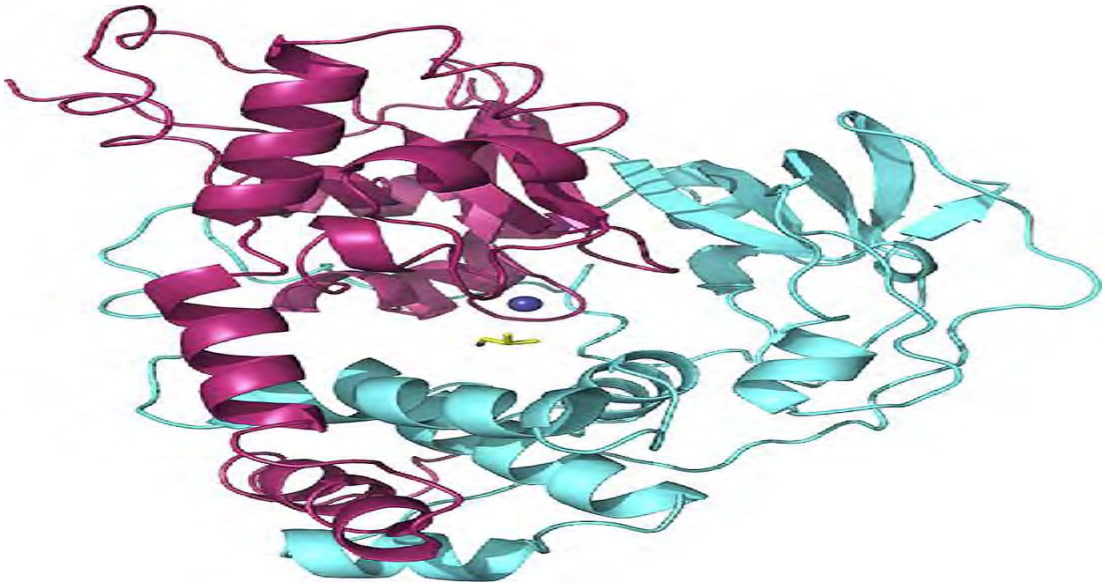


Figure 1.5: Ribbon representation of αβ heterodimer of nitrile hydratase from *Rhodococcus erythropolis* AJ270 reproduced from Song *et al.* (2007).

The magenta and the cyan ribbon represent α and β subunits, respectively. The yellow structure represents a substrate in the active site of Nhase and the blue sphere in the figure presents Fe^{3+}.

The two genes for the nitrile hydratase subunits are usually found as part of a gene cluster which also includes an amidase and regulatory protein-encoding genes and are generally induced by their amide products rather than their nitrile substrates.

Distribution: Nitrile hydratases are mainly found in bacteria (Banerjee *et al.*, 2002). They are reported to occur in species belonging to phyla *Proteobacteria*, *Actinobacteria*, *Cynaobacteria* and *Firmicutes* in habitats ranging from soil, coastal sediments, and deep sea sediments (Layh *et al.*, 1997).

Reaction Mechanism: Two possible types of general reaction mechanisms for nitrile hydratase are reported in the literature (figure 1.6). These mechanisms are described as first-shell and second-shell mechanisms (Van Pelt, 2010). The first-shell mechanism involves binding of the substrate nitrile to the ferric iron and the nucleophillic attack on the nitrile carbon by a water molecule activated by sulfenate group of αCys^{114}-SOH

(Hopmann *et al.*, 2007; Hashimoto *et al.*, 2008). In the second–shell mechanism, the hydroxide ligand from the ferric iron is activated and attracts the carbon atom of substrate nitrile. The hydroxide ion is believed to be formed by proton transfer to αCys^{114}-SO^{-} (Hashimoto *et al.*, 2008; Van Pelt, 2010).

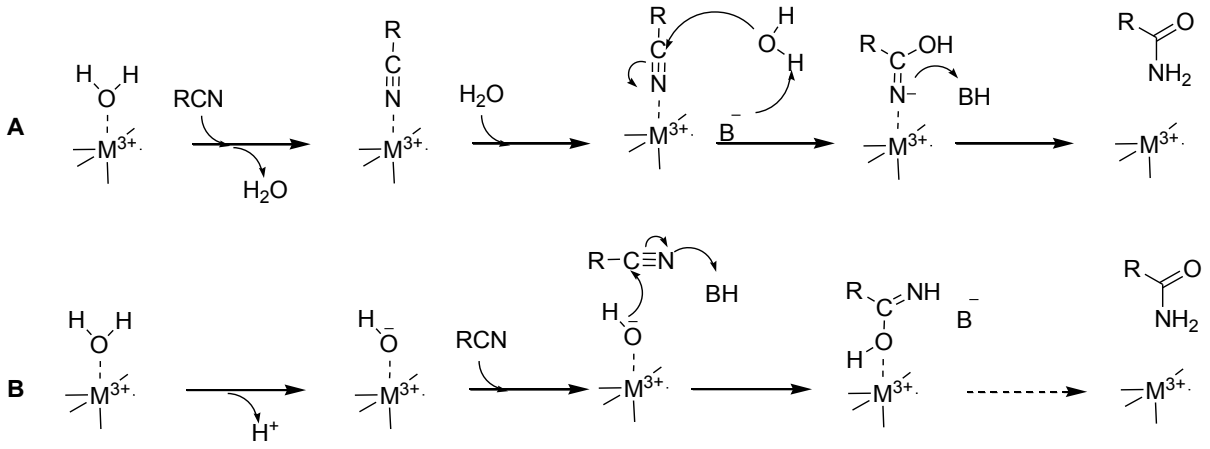


Figure 1.6: Proposed catalytic reaction mechanism of nitrile hydratase.

In the first-shell mechanism **(A)**, the nitrile substrates coordinates directly to the metal, while in second-shell mechanism **(B),** the metal hydroxide ion performs direct nucleophilic attack on nitrile substrate carbon (after Van Pelt, 2010).

Commercial Applications: Nitrile hydratase enzymes have considerable practical importance as biocatalysts for the industrial production of acrylamide and nicotinamide, and removal of nitriles from wastewater. For example, a nitrile hydratase expressing biocatalyst (*R. rhodochrous* J1) is used for the commercial production of acrylamide (propeneamide). The chemical biotransformation is performed on an industrial scale using whole cells of *R. rhodochrous* J1 with high nitrile hydratase activity. Mitsubishi Rayon manufactures 65,000 tons of acrylamide annually in this manner with a yield above 99.9%. Acrylamide productivity exceeds 400 g.l^{-1}.h^{-1} in this fed-batch reaction and the product concentration can reach 600-700 g.l^{-1}as it is insoluble and precipitates out of solution. Local production of acrylamide is also performed using a similar organism at Senmin (AECI, South Africa) at the multi-ton scale.

Using the same biocatalytic *R. rhodochrous* J1, Lonza produces over 3,500 tonnes of nicotinamide per year (Shaw *et al*., 2003) in China from 3-cyanopyridine with close to 100% yield, and a volumetric yield of 1,465 $Kg.l^{-1}$. These high yields are achieved as the biocatalyst may be added to a concentrated solution of 3-cyanopyridine providing a nicotinamide product of pharmaceutical quality that is subsequently spray-dried.

1.2.3. Amidase (EC 3.5.1.4)

Amidases catalyse the hydrolysis of amides to free carboxylic acids and ammonia (figure 1.7A), and are involved in nitrogen metabolism in both prokaryotic and eukaryotic cells (Banerjee *et al.*, 2002; Chen *et al*., 2009). In addition, the amidase from *Rhodococcus* R312 has been shown to catalyse the acyl transfer from a wide range of amides to hydroxylamine by Fournand and Arnaud (2001) (figure 1.7B)

Besides, amidase is also capable of catalyzing diverse reactions such as ester hydrolysis, hydroxamic acid hydrolysis, acid hydrazide hydrolysis, amide transfer onhydrazine, ester transfer on hydroxylamine, ester transfer on hydrazine and so on (Chen *et al*., 2009).

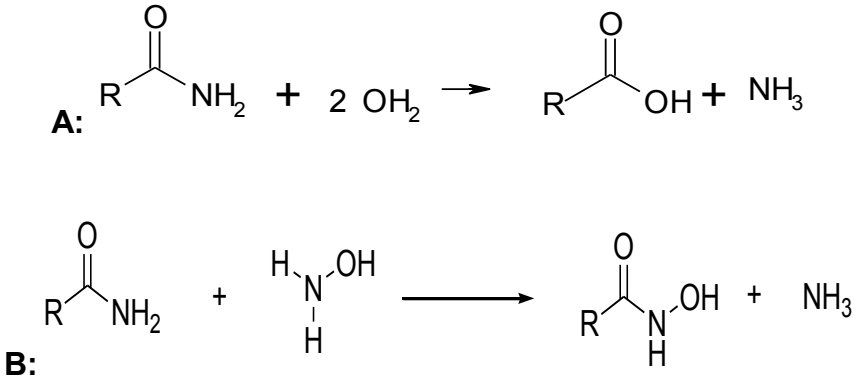


Figure 1.7: Amide hydrolysis (A) and acyl transfer (B), both by amidase.

Enzyme structure: Amidases have different quaternary crystal structures, organised either as homodimers, homooctamers, or homotetrameic. The crystal structure of *Pseudomonas aeruginosa* amidase showed a conserved two-fold symmetry αßßα-αßßα fold, similar to that of nitrilase (Novo *et al*., 2002). *Geobacillus pallidus* RAPc8 amidase quaternary studies by Kimani *et al*. (2007) revealed that the enzyme is a homo hexameric in solution and its monomers have typical nitrilase-superfamily αßßα fold. Amidase signature enzymes possess a unique, highly conserved Ser-Ser-Lys catalytic triad used for amide hydrolysis, although the catalytic mechanism for acyl-enzyme intermediate formation can differ between enzymes (Valiña *et al*., 2004)

Substrate specificity: Amidases display huge differences in substrate specificity and characteristics. For example, Kobayashi *et al*., (1999) reported the synthesis of hydrazide from hydrazine using amidase from *R. rhodochorus* J1 as a catalyst which they found to have unique characteristics of converting benzoic acid in the presence of hydrazine, to benzoic hydrazide and the reaction to be reversible. In addition, Fournand *et al.* (1998) used *Rhodococcus* R312 to catalyse the acyl transfer from a wide range of amides to hydroxylamine.

Reaction mechanism: The mechanism of reactions, amide hydrolysis *and* acyl transfer, catalysed by amidase is reported to be the same in literature (Banerjee *et al.*, 2003; Chen *et al.*, 2009). The possible mechanism of reaction catalysed by amidase occurs according to a Ping Pong Bi Bi type. The mechanism involves a nucleophillic attack by amidase on the carbonyl group of the amide, resulting in the formation of a tetrahedral intermediate, which is further converted to an acyl–enzyme intermediate with the release of ammonia (figure 1.8). The acyl–enzyme complex is then attacked by water or hydroxylamine resulting in the production of acid and a regenerated amidase.

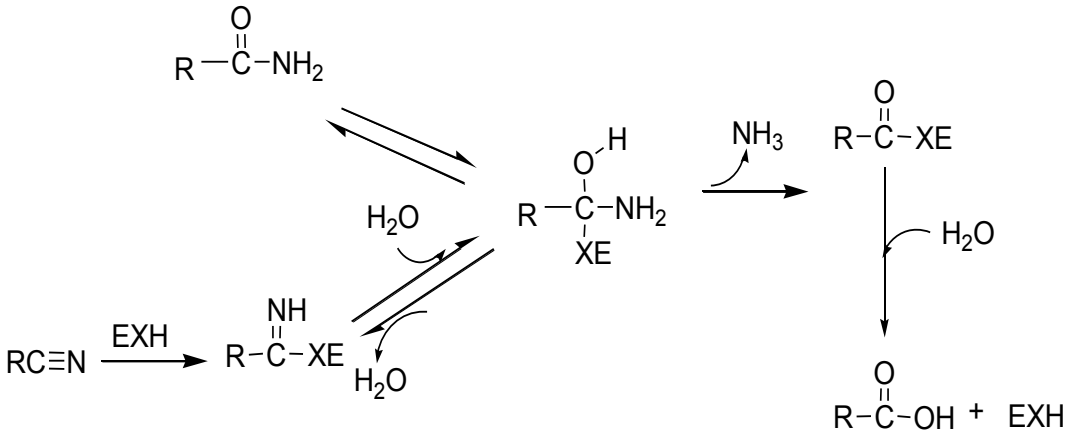


Figure 1.8: Mechanism of amidase-catalysed reaction
(reproduced from Chen *et al*., 2009).

Classification: Amidases are generally divided into two major distinct groups based on the amino acid sequence similarity. The first group includes aliphatic amidases, which hydrolyse only short-chain aliphatic amides (Asano *et al*., 1982; Hirrlinger *et al*., 1996) and the second group of aliphatic amidases which hydrolyse mid-chain length amides, and some α- or ω-amino amides (Stelkes-Ritter *et al*., 1995).

The aliphatic amidases belong to the nitrilase superfamily, and their catalytic residue is Cys which is believed to act as the catalytic nucleophile (Makhongela *et al.*, 2007). Most of the nitrilase superfamily have branches (Pace and Brenner, 2001; Novo *et al*., 2002). However, there are other amidases that are unrelated to the nitrilase superfamily (Brenner, 2002), such as amidase signature enzymes (Banerjee *et al.*, 2002), triad hydrolyases (Patricelli and Cravatt, 2000), and thiol proteases (Makhongela *et al.*, 2007).

The amidase signature enzymes belong to a group of amidases containing a *GGSS* signature in their amino acid sequence (Chebrou *et al.*, 1996). These enzymes are reported to contain asparagine and serine in the active site (Kobayashi *et al.*, 1998) instead of a Cys residue (Novo *et al.*, 1995), and have demonstrated enantioselectivity.

Application: Amidases are applied as industrial catalysts in the treatment of industrial effluents containing toxic amides, in organic synthesis, and in production of valuable therapeutic intermediates (Madhavan *et al.*, 2005). For example, nitrile hydratase coupled with amidase in organisms, *Rhodococcus* sp. C311 and *Rhodococcus erythropolis* MP50, have been applied in the enzymatic synthesis of (*S*)-naproxen from racemic naproxen nitrile to *S*-naproxen amide and subsequently to *S*-naproxen [S-2-(6-methoxy-2-naphthyl)propionic acid with enantiomeric excess above 99% (Effenburg and Graef, 1998). Komeda *et al*. (2004) purified an L-amino acid amidase from *Pseudomonas azotoformans* IAM 1603 that can biocatalyse (R, S)-piperazine-2-tert-butylcarboxamide stereoselectively to produce (S)-piperazine-2-carboxylic acid, which can be used for the synthesis of the HIV protease inhibitor *Crixivan*.

1.3. Biocatalysis monitoring

Monitoring of nitrile biocatalysis is important in biocatalytic and bioremediation process, as it can provide useful engineering guidelines towards optimal and/or successful bioproduction of the molecules of interest (Yang *et al.*, 2006).

There are a number of methods available for monitoring nitriles, carboxylic acids and amides. Most of the nitrile biocatalysis monitoring methods currently used are based on measurement of the ammonia formed from nitrile hydrolysis. Kaul *et al.* (2004) described measurement of nitrilase activity in several microorganisms using a pH-sensitive indicator-based colorimetric assay. This method is of limited applicability due to poor sensitivity, and the possibility of false reaction due to the generation of metabolic acids by the microorganism (particularly yeasts). Banerjee *et al*. (2003) described a fluorimetric assay method for the measurement of nitrilase activity, in which 3-cyanopyridine was hydrolysed to nicotinic acid using *R*. *rhodochrous*.

Fourier-transform infra red (FTIR) technology has been used to monitor nitrilase-catalysed reactions in real-time kinetics using a silicone probe (Dadd *et al.*, 2000). A major drawback to the FTIR methodology is limited availability of the type of instrument and silicone probe in most biological laboratories. However, the technique may provide kinetic data for nitrile hydrolysis.

There are also chromatographic methods (HPLC, GC, TLC, GC- or HPLC-MS) available for monitoring nitrile biotransformation. Reverse-phased liquid chromatography (RP-HPLC) coupled with UV detector has been used for determination of aromatic and unsaturated aliphatic nitriles and their reaction products. For example, determination of benzonitrile, benzamide and benzoic acid was achieved by RP-HPLC technique on a monolithic column (Brady *et al.*, 2004; Martínková *et al.*, 2008). Gas Chromatography (GC) coupled with a flame ionization detector is used for determination of saturated aliphatic nitriles or their corresponding amides and acids, since they are volatile. Thin layer chromatography (TLC) is a semi-quantitative technique performed on a support (glass, paper, plastic, and so on) coated with a thin layer of adsorbent material such as cellulose, silica gel, aluminium oxide, and other materials. This technique is used routinely by researchers to monitor the reactions, identify components in a compound mixture such as alkaloids, phospholipids, amino acids, and so on. Similar to other chromatographic techniques, TLC is based on the principle of separation.

1.4. Chiral separation

Chiral separation is an analytical component for drug discovery and development in the pharmaceutical, agrochemical, biotechnology, and other areas of natural products chemistry (Cavazzini *et al*., 2011). Drug-receptors are highly stereoselective with one enantiomer of the racemic pair providing the desired pharmacological activity, while the other enantiomer may exhibit negative effects (Krstulovic´, 1989, Kamal *et al.,* 2005b, Nguyen *et al*., 2006). Therefore, chiral discrimination and knowledge of enantiomer purity are important. The rapid introduction of optically active medicinal drugs and

pesticides, along with increasing government regulation, necessitates that rapid, sensitive and reliable stereochemical methods be devised for their analysis. This requirement has spurred a wave of activity in two ways: first, in the development on analytical procedures to quantify drug enantiomers and secondly, in the synthesis of single enantiomer drugs (Krstulovic´, 1989).

Among all the chiral separation techniques, chiral high performance liquid chromatography has proven to be one of the most robust and widely applicable platforms for the separation and analysis of enantiomers (Armstrong and Zhang, 2001; Gübitz and Schmid, 2001; Ward and Baker, 2008). In addition, it is more versatile than most other separation techniques and enables the successful resolution of a wide range of organic compounds, including most drug substances and intermediates. Current chiral HPLC methods are either indirect or direct.

1.4.1. Indirect method of separating enantiomers

In the indirect method, a racemic mixture is reacted with a chiral derivatising agent to form diastereomers which are chromatographed using an achiral liquid chromatography method. Because diastereomers possess different physiochemical properties, they can be separated in a non-chiral environment. The advantages of the indirect approaches are:

(i) less expensive, that is, conventional chromatographic columns can be used,
(ii) flexibility, because various non-chiral columns and mobile phases can be used,
(iii) numerous derivatising agents are available and the cost of each reagent may be less than non-chrial column, and
(iv) different selectivities can be achieved.

The disadvantages of using the method are:

(i) long analysis time that include sample preparation and verification of the derivatisation chemistry,
(ii) the need to synthesize non-commercially available derivatising reagents, and

(iii) possibly biased results for enantiomeric composition due to the racemisation of derivatising agent and unequal reaction rates (Souter, 1985).

1.4.2. Direct enantioseparation

Direct enantio-separation utilises chiral stationary phases (CSP) and chiral additives in the mobile phase. CSPs may be classified in many different ways. Wainer (1987) proposed five types of CSPs based on their interaction mechanism with the solute. There are other classification formats, such as chiral selector source (Armstrong and Zhang, 2001), and chemical characteristics (Cavazzini *et al.*, 2011) cited in the literature. Among the HPLC CSPs commercially available, only some types are presently most widely applied in industry: brush or Pirkle; crown ether based; cyclodextrin (CD); ion- and ligand; macrocyclic antibiotics; polysaccharide based; and protein.

Brush or Pirkle: The Pirkle-concept chiral stationary phases generally fall into three classes:

- π-electron acceptor/ π-electron donors
- π-electron acceptor, which is N-(3,5-dinitrobenzoyl)-phenylglycine bonded to n-propylamino silica
- π-electron donors (typically naphthyl-amino-acid derivatives covalently bonded to silica).

Major binding sites on Pirkle phases are classified as π-basic or π-acidic aromatic rings, acidic sites, basic sites and steric interaction sites. Enantiomeric separation is achieved by the following interactions:-

i. π-π interactions between π-donor and π-acceptor aromatic rings of racemic analyte and CSP,
ii. hydrogen bonding involving secondary amines and carbonyl groups on the CSP, with acidic proton, hydroxyl and amino groups on the racemic analytes,
iii. dipolar interactions as in dipole stacking, and

iv. steric interactions arising from non-polar bulky substituents attached to the stereogenic centre of the CSP that provide conformational control (Pirkle and McCune, 1989).

Racemic mixtures with polar functional groups must be derivatised so that they do not interact too strongly with the CSP. Derivatisation involves conversion of a hydroxyl group into an ester or carbamate, an amino group into an amide, urea or carbamate and a carboxyl into an ester or anilide.

Available columns include: Whelk-O 1, Whelk-O 2, ULMO, DACH-DNB (mixed phase, α-Burke 2, β-Gem 1 (π-acceptor phases), Naphtylleucine (π-acceptor phases) (all from Regis Technologies, Inc , Morton Grove, Illinois, USA) and Phenomenex Chirex phases (from Phenomenex, St. Torrance, California, USA).

Whelk-O 1 chiral stationary phase was originally developed by Pirkle (Pirkle and Finn, 1981) for separating the enantiomers of naproxen (figure 1.9). This CSP contains both π- acid group (3, 5-dinitrobenzoyl) and π-base group (naphthyl).

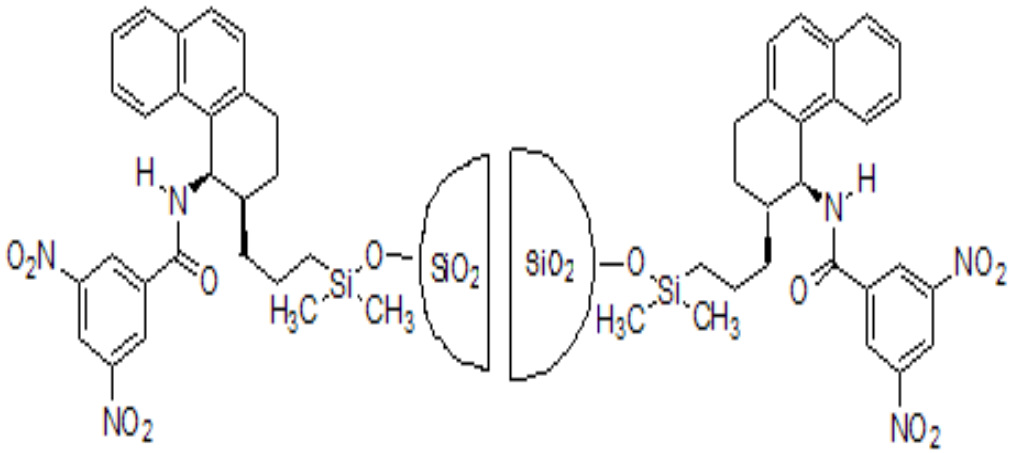


Figure 1.9: Whelk-O 1 chiral stationary phase

(reproduced from Ahuja S., 2008).

The advantages of the brush CSPs include: good selectivity; compatibility with a broad range of solvents; stability; inversion of the elution order; availability; and low cost. The disadvantages are that the CSPs only work with aromatic compounds, and that derivatisation may be required to aid separation.

Crown ether based:

Chiral crown ether-based CSP are synthetic macrocyclic compounds with the appearance of a crown-like cavity wherein enantioselectivity take place. The chirality in the crown ether is developed by introducing crown ether oxygens which act as electron donor ligands (Cavazzini *et al.* 2011), inside the wall of the cavity.

Crown ether chiral mechanism is based on inclusion phenomena, which involves complex formation between the crown ether oxygens and ammonium cations from the sample that fit well into the cavity of CSP. For example, Cho *et al*. (2009) prepared a new crown ether based CSP containing the thioester linkage by bonding (+)-(18-crown-16)-2,3,11,12-teracarboxylic acid to mercarptopropylsilica gel (figure 1.10). This new CSP is reported to have a better chiral ability as compared to previous CPSs containing the amide linkage for resolving various α-amino acids, racemic amines and amino alcohols.

The most commonly used crown ether is “18-crown-6 cyclic” which has been made available commercially as the “Crownpak Crown ethers (+) or (-) by Daicel Chemical Industries Ltd., Tokyo, Japan. These CSPs are efficient in enantioseparation since they operate opposite to each other (that is, reversed elution order).This capability renders them favourable in trace analysis of unwanted enantiomer. The disadvantages of crown ether based CPSs are: limited applicability, high cost, and mobile phase (that is, aqueous acidic) restriction.

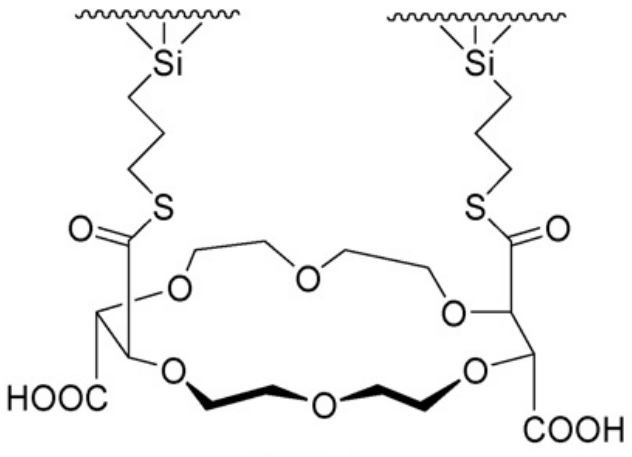


Figure 1.10: Structure of (+)-(18-crown-6)-2,3,11,12-tetracarboxylica acid bonded to mercarptopropylsilica gel

(reproduced from Cho *et al*. 2009).

Cyclodextrin (CD):

Cyclodextrins are cyclic oligosaccharides consisting of α-(1-4) glycosidic linked D(+) glycose units. CDs can be described as truncated cones where all secondary hydroxyl groups are directed towards the larger opening, and smaller opening at the other end is formed by the primary hydroxyl. The native CD is primarily immobilised via one or two C2 or C3 hydroxyl group. The remaining hydroxyl can be free or derivatised. The derivatisation of these groups will influence the geometric accessibility of the chiral cavity and the binding strength of the complex by improving the steric fit.

The primary mechanism of interaction between chiral selector and analytes is the inclusion of hydrophobic molecules into non-polar cavity of the CD. The diastereomeric inclusion complexes formed, are based on hydrophilic interaction (between the cavity and guest molecule) and stereoselective hydrogen bonds (between C2 or C3 hydroxyl groups of glucose and guest molecule), finally leading to a separation of enantiomeric mixture (Ward and Armstrong, 1986).

Alpha-, β- and γ- cyclodextrins are the most used and available commercially from either Advanced Separation Technologies Inc. (ASTEC, Whippany, New Jersey, USA) or JM Sciences (USA); they contain 6, 7, and 8 glucose units, respectively (Cyclobond Handbook, 2005). One of the major advantage of the CD based CSP is their successful use in normal-, reverse-phased, polar organic and polar ionic mode of mobile phase. These CSPs are limited by inclusion phenomena; analyte must have hydrophobic or aromatic group to fit into cavity.

Ligand exchange:

Chiral ligand exchange chromatography has been widely employed in resolving α-amino acid enantiomers since the pioneering work of Danakov and Kurganov. The mechanism of this separation is based on the formation of ternary complexes between the analyte divalent metal cation and the chiral selector. In the first case, the diastereomeric complexes are formed in the bulk of the mobile phase and then they are adsorbed on achiral stationary phase. In the second case, enantioselective formation of ternary complexes between the chiral phase and the analyte plays the main role. In both cases the stereoselective recognition results from small energy differences in the chiral complex stability. The enantiomer which forms the most energetically-stable complex with the CSP will be the one that is retained longer. The observed enantioselectivity of the chromatographic system depends on the CSPs surface, the concentration of the metal ion, and other modifiers in the mobile phase. The most popular chiral selectors, α-amino acids and their N-substituted derivatives, are usually applied as chiral mobile phase additives, or as chiral molecules of the stationary phase after binding covalently or hydrophobically, to a support (Rogozhin and Danakov, 1971).

Macrocyclic antibiotics:

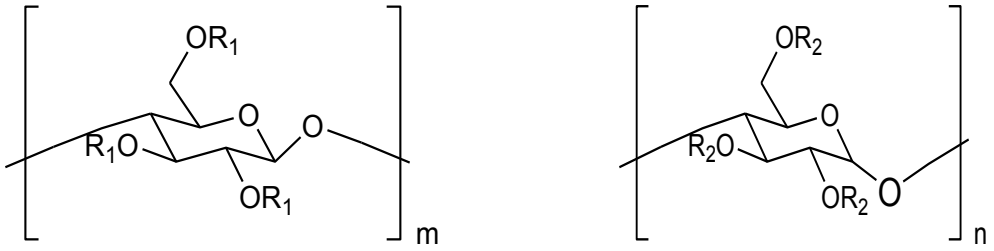
Macrocyclic antibiotics based CSPs are prepared by immobilising themacrocyclic glycopeptides onto the silica. Glycoproteins such as ansamycin, avoparcin, teicoplanin, ristocetin A, as well as the polypeptide thiostrepton have been used through immobilisation onto silica (Ilisz *et al*. 2006; Cavazzini *et al.*, 2011). They possess several characteristics that allow them to interact with analytes and serve as chiral

selectors (Ilisz *et al*. 2006). They have a number of chiral centers within cavities for chiral molecule to enter and interact with various functional groups (Okamoto and Ikai, 2008; Cavazzini *et al.*, 2011). The interactions include: Van der Waals interactions, π-π complexes, hydrogen bonding, ionic interactions, inclusion complexation, steric interactions, and others.

Macrocyclic antibiotics CSPs are capable of running in RP-HPLC, normal phase, polar organic and polar ionic modes. The available columns are: Chirobiotic V and V2 (Vancomycin), Chirobiotic T and T2 (Teicoplanin), Chirobiotic R (Ristocetin A) from ASTEC.

Polysaccharide based:

Polysaccharides based CSPs are produced by physically coating of the polysaccharide derivatives on a silica matrix (Okamoto *et al.*, 1984). Polysaccharide derivatives, such as cellulose and amylose, are known to show high chiral recognition abilities for many racemates when used as CSPs (Cavazzini *et al.*, 2011). The cellulose derivative contains β-D-1-4-glycoside linkage and forms a linear structure, while the amylase derivative CSP contains α-D-1-4-glycoside linkage and adopts a helical structure. The structures of the functional groups for these CSPs are shown in figure 1.11.



A **B**

Figure 1.11: Structure of cellulose (A) and amylose (B) derivatives for polysaccharides based chiral stationary phase

R_1 = tribenzoate or tris(3,5-dimethylphenylcarbamate); R_2 = tris(3,5-dimethylphenylcarbamate) (after Tachibana and Ohnisini, 2001)

The separation mechanism of these CSPs involves interaction of the polysaccharide derivative functional groups with those on chiral analytes. These interactions are:

- dipole–dipole stacking interaction
- hydrogen bonds
- π-π stacking interaction

These interactions are relatively weak and are considered to be more effective under normal phase HPLC conditions (Tachibana and Ohnisini, 2001). The chiral recognition is also a function of fit of the asymmetric part of the analyte into the chiral cavity of the chiral stationary phases (Chiral Technologies, 2003). Thus, not only the electronic, but the steric properties of certain chiral compounds and that of polysaccharide based CSPs have to be taken into consideration.

Polysaccharide CSPs with cellulose and amylase backbone have a wide range of application. For example, Wu and Li (2003) resolved hydrolysis products of 3-hydroxy-4-arylbutanenitriles. Winkler et al. (2005) used Chiralpak AD-H and Chiracel OD-H in their studies on the synthesis and microbial transformation of β-amino acids with *Rhodococcus erythropolis* NCIMB 11540 and *R. equi* A4.

The disadvantage of coated polysaccharides CSPs is the limitation of the solvents that can be used in mobile phase and samples dilution. This limitation was addressed by introduction of new immobilised CSPs (Chiralpak IA, and IB) which use the same chiral selectors as AD and OD, respectively, but these are immobilised on the silica; more robust and have much greater solvent compatibilities. The first new generation of immobilised polysaccharide-based CSP is CHIRALPAK® IA which utilizes 3,5-dimethylphenylcarbamate of amylose and which can function in a wide range of solvents other than alcohols (Zhang *et al*., 2005).

Protein based CSPs:

Protein based CSPs are produced by immobilising natural protein on a silica support. Proteins are polymers made of α-amino acids as the monomeric unit and contain large

number of chiral centers that interact strongly with small chiral molecules through: hydrophobic and electrostatic interactions, and hydrogen bonding. The CSP disadvantages include:

- it requires aqueous based conditions;
- analyate must have ionisable groups such as amine or acid;
- not suited for preparative applications due to low number of recognition sites;
- stability, some proteins readily change their conformation.

Protein based CSPs play a major role in bioanalytical monitoring of a chiral drug in the body because of their ability to discriminate chiral molecules (Haginaka, 2001).

The protein based CSPs used for chiral separation in HPLC include bovine serum albumin (BSA), human serum albumin (HSA), α_1-acid glycoprotein (AGP), ovomucoid from chicken egg white; avidin and cellobiohydrolase I (CBH) (Chromtech Ltd. Cheshire, U.K.; Regis Technologies, Inc., USA; Okamato and Ikai, 2008).

Protein CSPs are of special interest because they are suitable for separating a wide range of enantiomers. For example, D- and L-tryptophan were resolved using BSA (Allenmark, 1988). (S)-Warfin and (R)-Warfin were separated on HSA, and S-Warfin was eluted first; this elution order was opposite to that observed on BSA-based CSP (Domenici *et al.*, 1990).

Commercially available protein based CSP columns are: Chiral AGP (α-glycoprotein) from ChromTech, Inc., USA; HSA (human serum albumin) from ChromTech; BSA (bovine serum albumin) from Regis Technologies, Inc., USA.

CSPs are designed to give maximum selectivity in certain mobile phases. The critical factor in chiral separation is the required functionality present in any CSP to allow selectivity to occur. Many CSP developments focus on the ability to operate in different modes of separation (normal, reversed polar organic and polar ionic mode) due to a wide range of molecules and polarities (Allenmark, 1988).

To date nearly a hundred HPLC CSPs have been developed and are commercially available, including Pirkle type, ligand-exchange, cyclodextrin, crown-ether, macrocyclic glycopeptide, protein, chiral polymers and polysaccharide-based CSPs (Cavazzini *et al.*, 2011). However, there is no single CSP that has the ability to separate all classes of racemic compounds, because most chiral HPLC columns used in chiral separation are specific. Choosing the right chiral HPLC column for the enantioseparation of a chiral compound is difficult. Most of the separations achieved are based upon the accumulated trial and error knowledge of the analyst, chiral method development is sophisticated, time and resource consuming. In addition chiral mechanisms are complex and not fully understood.

1.5. Enzyme Immobilisation

Enzyme immobilisation may be defined as associating the enzyme molecules with an insoluble matrix, so that it can be retained in a proper geometry for its economic reuse under stabilised conditions. Materials used for immobilisation of enzymes, called carrier matrices, are usually inert polymers or inorganic materials.

Enzymes are known to display advantageous catalytic properties. However, other critical enzyme properties need enhancement, including stability, activity, and selectivity towards non-natural substrates (Mateo *et al*., 2007). Immobilisation of enzymes is one of the methods used to solve these limitations; it can increase operational stability and enhance the rigidity the enzyme structure. For example, improvement in lipase activity and enantioselectivity has been achieved by immobilisation of the enzyme.

Types of enzyme immobilisation: There are three types of methodology for enzyme immobilisation: binding to a support (carrier); inclusion/ entrapment or encapsulation in an inorganic polymer; and cross-linking of protein molecules (Sheldon, 2007; Matijošytė *et al.,* 2010).

1.5.1. Support binding

The carrier selected for this kind of immobilisation technique depends on the nature of the enzyme. However, the following aspects regarding the carrier are important: particle size; surface area; molar ratio of hydrophilic to hydrophobic groups and chemical composition (Cao, 2005a). Support binding can be sub-divided into physical adsorption, ionic binding and covalent binding (Cao, 2005a).

(i) **Physical adsorption**: Physical adsorption is the net result of various types of interaction in which the enzyme (e.g. lipase), the support material and water or other solutes are involved. Electrostatic and hydrophobic interactions contribute immensely to the adsorption of the enzyme to the support material (Cao, 2005a).

Electrostatic interactions: Interactions between charged groups and/or dipoles of lipase and support material (for example, cellulose) may occur. Such interactions influence the affinity of the protein to the surface (Geluk *et al.,* 1992).

Hydrophobic interactions: Dehydration of hydrophobic regions of both the protein and the support may contribute to the adsorption of the enzyme. As lipase acts at the oil-water interface, it is often stated that lipases have a hydrophobic region on their surface that upon adsorption orients towards the oil interface (Geluk *et al.,* 1992).

The physical binding technique is advantageous in that little or no conformational change occurs to the enzyme, nor is the active site chemically inactivated. However, the enzyme may break away (“leak”) from the carrier during utilization because of weak binding force between enzyme and the carrier (Brady and Jordaan, 2009; Sheldon, 2007). Inorganic materials such as alumina, silica, amberlite XAD-7, zeolites, porous glass, have been used as carriers of enzymes (Petri *et al.,* 2005; Takaç and Bakkal, 2007; Sheldon, 2007).

(ii) **Ionic binding:** This binding is based on the ionic interaction of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Comparison of this technique to others is shown in table 1.1.

(iii) **Covalent binding:** Covalent binding technique is based on the interaction of enzymes and water-insoluble carriers by covalent bonds. The functional groups of the enzyme‟s amino acids can be used in the covalent interactions between enzyme and the carrier. These functional groups could be:

- ε amino group
- α, β, or γ carboxyl group
- sulfhydryl group
- hydroxyl group
- imidazile group
- phenolic group

These functional groups react with the carriers containing reactive groups such as diazonium, acid azide, isocynates and halides. Reaction conditions required for covalent binding are relatively complex and not particularly mild. The binding between the enzyme and the carrier is very strong and more difficult to break than in the case of physical adsorption and ionic binding (Sheldon, 2007).

The most prevalent and applicable commercially available matrices for covalent coupling of enzymes are polysaccharides, agarose, cellulose and their derivatives. Agarose, calcium aliginate and carrageenan, are gel-forming agents widely used for whole cell and enzyme entrapment. Gel-forming agents are hydrophilic polymeric materials which swell in water while maintaining a three dimensional structure (Wichterle and Lím, 1960).

Table 1.1: Generalised comparison of different enzymes immobilization techniques

Characteristic	Physical adsorption	Ionic binding	Covalent binding	Entrapment	Cross-linking
Preparation	easy	easy	difficult	difficult	intermediate
Enzyme activity	low	high	high	high	moderate
Substrate specificity	unchangeable	unchangeable	changeable	unchangeable	changeable
Binding force	weak	moderate	strong	strong	strong
Regeneration	possible	possible	impossible	impossible	possible
General applicability	wide	moderate	selective	wide	moderate
Cost of immobilisation	low	low	high	low	moderate
Reference	Sheldon, 2007; Brady and Jordaan 2009,	Cao, 2005a.	Mateo et al., 2000; Pierre *et al.* 2006	Lalonde and Margolin, 2002; Liu *et al.*, 2009;	Brady and Jordaan 2009,

Resyn^{TM} (a dendrisphere) is an organic polymer based resin with relatively high functional group density, with a loose backbone and interpenetrating network that allows for high enzyme loading as compared to other supports (Brady and Jordaan, 2009). This technology employs the formation of emulsion of oil droplets containing polyethyleneimine (PEI) dispersed in the same phase containing glutaraldehyde resulting in a dendritic polymeric backbone. The particles are easily recovered from the emulsion after which they are used for protein immobilisation (Jordaan *et al*., 2009a). Exposed functional groups on the particles are subsequently used to bind proteins through covalent interaction with the primary amine groups on the enzymes surface through a Schiff base reaction (Harris *et al,* 1984; Roberts *et al.,* 2002). The

dendrisphere technology is advantageous in that enzyme loading is high and enzyme loss is reduced when compared with other solid support immobilisation media, and use of an emulsion technology allows particle uniformity and monodispersity. However, it may suffer from the major disadvantage of enzyme self-immobilisation techniques, namely substrate diffusion constraints (Brady and Jordaan, 2009). The dendrisphere support material is marketed as Resyn^{TM} by Resyn Biosciences (http://www.resynbio.com/index.htm).

1.5.2 Entrapment

Entrapment is based on confining the enzyme in the lattice of a polymer matrix such as an organic polymer or a silica sol-gel or enclosing enzyme in semi-permeable membranes (Sheldon, 2007). The bonding between the enzyme molecules and the matrix is non-covalent, and the entrapment may be envisaged as an enzyme molecule in a molecular cage (figure 1.12). For example, horseradish peroxidises and chloroperoxidase were entrapped in a nanosphase-separated amphiphilic network made of a co-polymer of poly (2-hydroxyethyl acrylate) (PHEA) and bifunctional poly (dimethylsilicoxane) (PDMS) (Bruns and Tiller, 2005).

Entrapment is one of the most important techniques used to immobilised cells. A wide variety of cells had been encapsulated in alginate polymer hydrogel beads for different application (Wong and Chang, 1991; Yang *et al.,* 2002). Enzymes may also be entrapped, but this requires different polymers with smaller pores. Encapsulation helps segregate the cell or enzyme from the adverse environment. The major disadvantage of entrapment technique is high porosity of the matrices that result in slow enzyme leakage (Liu *et al.,* 2009).

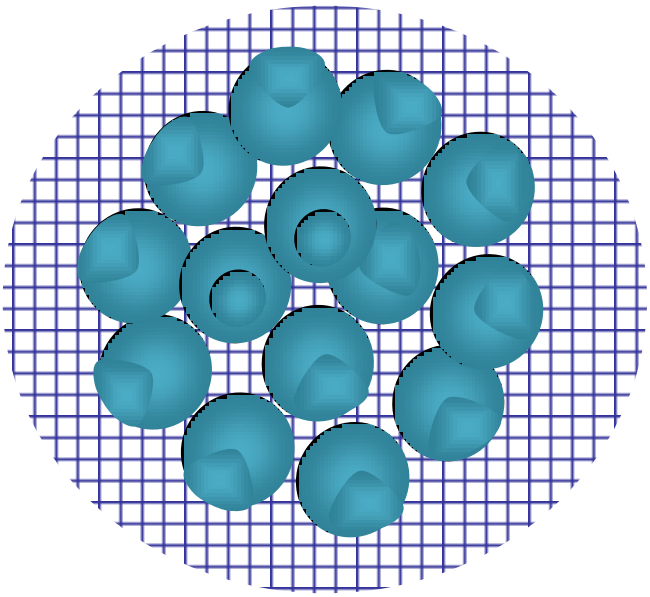


Figure 1.12: Entrapment methodology.

Enzymes are represented by circles (adapted from Brady and Jordaan 2009).

1.5.3 Self cross-linking

Enzyme self cross-linking techniques are based on the formation of covalent chemical bonds, but no support matrix is included. Immobilisation occurs through the intermolecular cross-linkages between enzymes molecules by means of bi- or multifunctional reagents. These bi-functional reagents are chemical modifications agents and may be classified as zero-length, homo-functional and hetero-functional cross-linkers. Such cross-linkers react with nucleophilic side chains of amino acids, such as the sulfhydryl group of cysteine (Cys), amino group of lysine (Lys) and N-terminal amino acids, carboxyl group of aspartic (Asp) and glutamic (Glu) acids and the C-terminal amino acids, imidazole group of histidine (His) and thioester group of methionine (Met). Zero-length cross-linkers induce direct joining of two cross-linked groups. Examples of zero-length cross-linkers include ethyl chloroformate, carbodiimide and 1,1-carbonyl diimidazole (Wong and Wong, 1992).

Cross-linking reactions are carried out under relatively severe conditions which can change the conformation centre of the enzyme, and lead to significant loss of activity

(Sheldon *et al.,* 2006). Homo-bifunctional cross-linkers are linkers that have identical reactive groups. Examples of such reagents include bis-imidoesters, bis-succinimidyl derivatives, di-isocyanates, di-isothiocyanates, di-sulfonyl halides, bis-nitrophenyl esters, di-aldehydes and di-acylazides (Wong and Wong, 1992).

Among cross-linking reagents, glutaraldehyde is the most commonly used for enzyme immobilisation (Wong and Wong, 1992; Cao *et al.,* 2003). However, due to its small size and reactivity, it can easily penetrate the protein and lead to deactivation (Chae *et al*., 1998). Cross-linking is also referred to as carrier-free enzyme immobilisation technique since it involves covalently linking of enzymes molecules to each other by a cross-linking agent(s) such as glutaraldehyde polymers, or glutaraldehyde elongated with ethylenediamine (EDA) or PEI (Cao *et al.* 2003, Brady *et al.* 2008). There are different approaches in carrier-free immobilisation technique. They include cross-linked enzyme crystals (CLEC), cross-linked enzyme aggregates (CLEA), cross-linked (dissolved or soluble) enzymes (CLE), cross-linked spray dried enzymes (CSDE), and SphereZymes^{TM} (SZ) (figure 1.13). These approaches have been elucidated to retain highly concentrated enzyme particles with no leakage of enzymes to the reaction medium as in physical adsorption (Cao *et al.* 2003; Brady *et al.,* 2008).

CLE are relatively cheap to produce and only require a cross-linking agent to randomly cross-link the dissolved or soluble enzyme molecules. Limited stability of the enzyme as a result of the difficulty optimising the amount of cross-linking agent during preparation and difficulties encountered in handling soluble CLE makes this technique unfavourable. The CSDE technique has not been exploited, as the spray-drying method often deactivated the enzymes.

CLEA are formed by precipitation and subsequent cross-linking. By changing properties that affect the proximity of soluble enzyme molecules such as the electrostatic constant of the enzyme solution, which is done by adding non-denaturing protein aggregation agents, enzyme aggregates are formed. These are subsequently cross-linked by a bi-functional cross-linker to form CLEA (Cao *et al.,* 2003). The CLEC technology is

analogous to CLEA, however it requires enzyme crystallisation, and hence enzyme of high purity is necessary (Haring and Schreier, 1999).

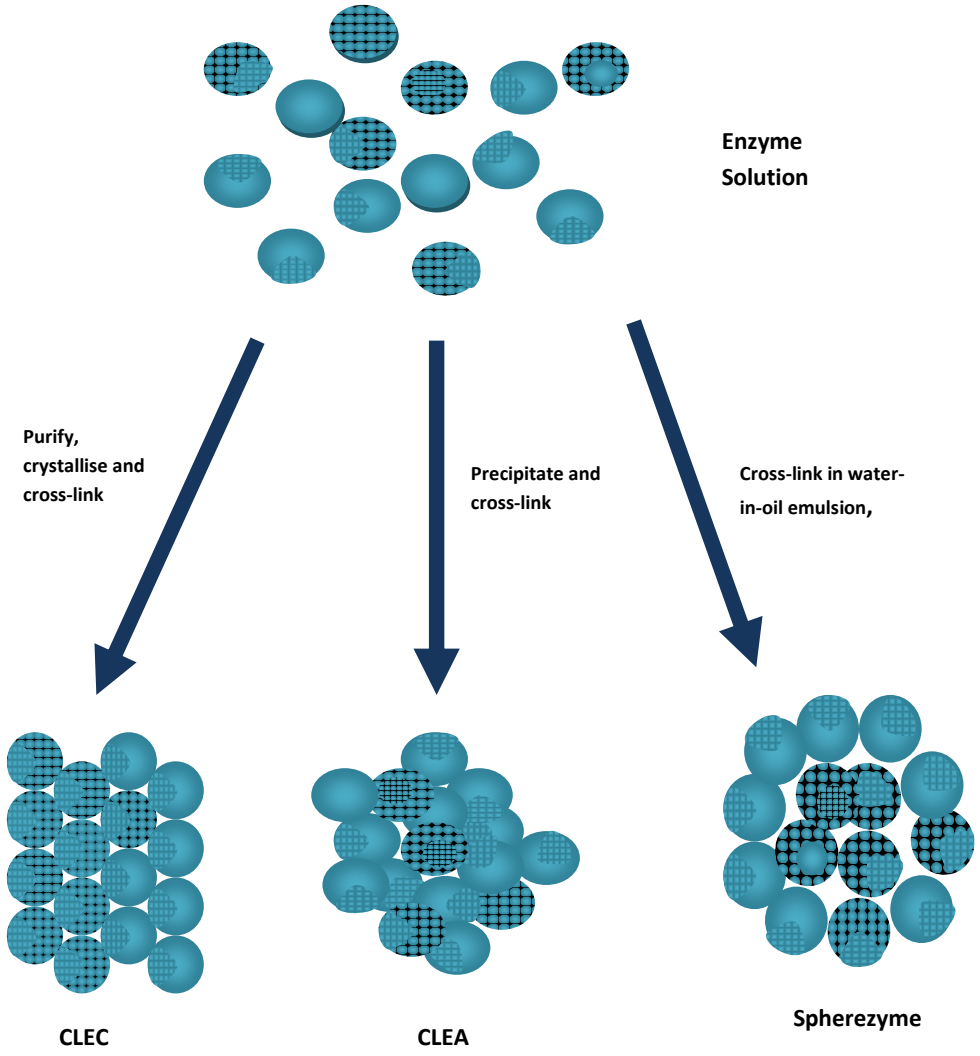


Figure 1.13: Comparison of some of carrier-free enzyme immobilisation
(adapted from Brady and Jordaan, 2009).

Although CLEC and CLEA technologies produce enzymes with enhanced conformational stability (temperature, pH, organic solvents), diffusion constrains encountered with increasing particle size of the immobilised enzyme makes the commercial applicability of these techniques limited (Brady *et al.,* 2008).

The major drawback of carrier-free immobilisation technique is that some of the active sites of the cross-linked enzymes are not exposed, and hence additional catalyst to compensate for a particular function is often required, increasing the operational cost. To compensate for the disadvantages encountered with carrier-free immobilisation, techniques combining carrier-binding and carrier-free immobilisation were developed (Cao, 2005b). In this method, it was demonstrated that CLEA could be prepared in preformed carriers. Although these methods provided combined advantages of carrier-free and carrier-bound techniques, in practice, they are not economically favourable.

1.6 Problem statement

The application of biocatalysis in chemical organic synthesis provides the analyst with a number of challenges. Firstly is that the reactions tend to occur in water, and hence any at-line analytical method should be water based, such as HPLC. Secondly, many applications of biocatalyst are for the synthesis or resolution of chiral compounds. In the latter case the enzymes are actually far better at discriminating between enantiomers than most analytical methods. Hence specific chiral separation columns are required in conjunction with specific elution methods.

In this study the nitrile hydratase activity of *Rhodococcus rhodochrous* ATCC BAA-870 (an organism isolated in CSIR Biosciences laboratories, South Africa) will be evaluated and its ability to catalyse both chiral and non-chiral compounds will be quantified using various HPLC methods. The enzyme will be used as a whole cell biocatalyst, as well as self-immobilised macroscopic enzyme particles.

1.7 Objectives of this study

The objectives of the study are as follows:

(i) To develop non-chiral and chiral HPLC methods for quantification of compounds present in various nitrile biocatalysis reactions;

(ii) To partially purify nitrile hydratase from *Rhodococcus* BAA-870 and quantify it using benzonitrile;

(iii) To show that partially purified nitrile hydratase can be immobilised using the SphereZyme™ technology;

(iv) To evaluate the application of whole cell nitrile hydratase active biocatalyst against β-substituted chiral compounds.

Chapter 2: Biocatalyst Activity Determination

2.1 Introduction

High-performance liquid chromatography (HPLC) is a solution-phase technique that can separate a mixture of compounds, and is used in analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC involves a pump that moves the mobile phase(s) and analytes through a column, which contains the so-called “stationary phase” in the form of densely packed, often chemically functionalized, beads and a detector that determines the characteristic retention time for the analytes. Different types of stationary phase, such as hydrophobic saturated carbon chains, are used according to the analytical challenge. Once analytes are introduced to the column, it is the sample‟s differential affinity for the specific solvent (“mobile”) and stationary phases that enables separation to occur, generally with sharper resolution than standard liquid chromatography. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

HPLC columns are available in a variety of formats, including reversed-phase, ion-exchange, chiral and size exclusion, which separate based on polarity, charge, and size respectively.

In this study, an HPLC method for separating and quantifying benzonitrile and its related acid and amide was developed using X-Terra MS (or RP) C18 3.5 µm column (Waters, Milford, MA, USA). This column has modified silica in which the stationary phase is a hybrid of silica and organosiloxane referred to as Hydbrid Particle Technology. This substitution greatly reduces analysis time, improves the peak shape of basic compounds and increases the stability of the stationary phase against extremes of pH and temperature (X-Terra Columns, 1999).

Brady *et al*. (2004) had previously reported that *Rhodococcus rhodochrous* ATCC BAA-870, a soil isolate, exhibited nitrile hydratase activity. The developed HPLC method of

separating benzonitrile and its corresponding hydrolysis products will be applied to the quantification of benzonitrile in determining the activity of *Rhodococcus rhodochrous* ATCC BAA-870 and its partially purified nitrile hydratase.

2.2 Aims of the method development

a) The aims of the RP-HPLC method development for the determination of benzonitrile and its related acid and amide using X-Terra MS (or RP) C18 3.5 µm column were:- To develop a simple and rapid method that could separate and accurately quantify benzonitrile and its related acid and amide with good baseline resolution and reduced run time. This could then be used to accurately quantify the activity of nitrile biocatalyst.
b) To optimize the developed method for reducing solvent use and waste.

2.3 Materials and Methods

2.3.1 Chemicals

Organic solvents, methanol (MeOH), hexane (Hex) ethyl acetate (EtOAc), dichloromethane (DCM) and acetonitrile (ACN) were of HPLC grade, and were purchased from Radchem C.C. (South Africa). Trifluorocetic acid (TFA), and benzonitrile were purchased from Sigma-Aldrich (St. Louis, MO). HPLC MilliQ water (18 Ωm) was obtained from a Milli-Q purification system (Millipore, USA)

2.3.2 Preparation of mobile phase

TFA (0.1%,w/v) was prepared by diluting 1 ml TFA with nanopure water to 1 l in a volumetric flask, and it was filtered through a 0.45µm pore nylon membrane (Millipore, USA) before use.

2.3.3 Instrumentation

The liquid chromatography system consisted of a Waters 2690 separation module coupled with a Waters Diode Array Detector 996 (Waters, Milford, MA, USA). Data integration was performed using Empower 2 software. The HPLC system was programmed in isocratic mode, using different eluents (table 7.1). UV-light adsorption was monitored at 10 nm intervals from 210 to 400 nm; the wavelength was set at maximum wavelength for each test to integrate the peaks. The injection volume was 10 µl, injected with an autosampler, column temperature fixed at 25°C and flow rate at 0.5 $ml.min^{-1}$.

The analytes were separated using X-Terra MS (or RP) C18 3.5 µm, 3.0 × 50 mm [internal diameter (i.d.) × length (L)] column. Benzonitrile and its corresponding amide and acid were separated isocratically by using eluent of 0.1% TFA (in MilliQ water) and acetonitrile at different composition as indicated in table 7.1. RP is a general purpose reversed phase column, while MS is designed to be compatible with mass spectrometry applications (and was used for runs wherein the compound identity was confirmed using mass spectrometry).

2.3.4 Preparation of stock standard solution

The standard stock solution of 10 mM benzonitrile mixed with its corresponding amide and acid was prepared using mobile phase made of 0.1% TFA in water and acetonitrile (70:30, %v/v), in a minimum amount of methanol (5% v/v). The standard stock solution was diluted with mobile phase solution to obtain working standard solutions between 0.0 and 3.0.mM.

2.3.5 Method Validation

2.3.5.1 Calibration Curve

Linearity is defined as the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. It is generally reported as the standard error and the correlation of the regression line used to quantify data (Green, 1996). Range is the (inclusive) interval between upper and the lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity using the methods. The range is normally expressed in the same units as the test results obtained by the method.

The linearity of this method was tested by preparing stock standards of benzonitrile, benzamide and benzoic acid (10 mM concentration). Standard solutions were prepared by diluting the stock standard solution with mobile phase solution to obtain concentrations in the range of 0.0 to 3.0 mM. Each preparation was analysed in triplicate. The linear regression analysis was performed and the curve plotted using Empower 2 software.

2.3.5.2 Precision

Precision consists of two components namely, repeatability and reproducibility. The repeatability of analytical method is the closeness of the data produced by the method when operated under normal conditions on the same instrument, in the same laboratory, by the same analyst (Green, 1996). It indicates instrument related variances and random analytical errors. Reproducibility is the closeness of the agreement between the results of measurement of the same measure carried out by different analysts on different instruments on different days or in different laboratories (Green, 1996). The reproducibility data indicates the ruggedness of the analytical method. Both forms of precision are expressed as percentage relative standard deviation (%RSD).

Method precision was performed by injecting nine 2.0 mM standard mixtures consecutively to calculate the %RSD on amount, peak area and amount of each compound used in the study. The average of the nine preparations with %RSD was automatically calculated by the software.

2.3.5.3 Accuracy

Accuracy is the measure of the exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted as a conventional, true value or an accepted reference value (Green, 1996). There are various ways of measuring accuracy:

- Standard reference material: Analyse a sample of known concentration (Standard Reference Material) and determined the mean of the results. Compare the mean with the true value.
- Recovery experiments: Spike a blank with a known quantity of the analyte at a concentration close to the quantification limit. Determine the recovery of known amounts of analyte spiked into sample matrix.

In this method, accuracy was determined by analysing sample of standard mixtures at concentrations of 1.0, 1.5 and 2.5 mM and comparing it with true value. This was done to check recovery of the method at different levels.

2.3.5.4 Limit of Detection and Quantification

Limit of detection (LOD) is defined, as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantified under method conditions. Limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method (Green, 1996).

LOD and LOQ are related to signal noise ration and are typically estimated statistically from the linear calibration ($y = mx + c$) using:-

LOD = c + 3 Sy/x,

LOQ = c + 10 Sy/x.

Where c = intercept from the calibration curve, and Sy/x is the standard error of the calibration graph. LOD and LOQ were obtained from the calibration curves of benzamide, benzoic acid benzonitrile standards.

2.3.6 Biocatalyst Preparation

Microorganism and cell cultivation

Strain ATCC BAA-870 was cultured in a rich media, tryptone soya broth (TSB) obtained from Anatech (Schatlau Chemie, S.A. Barcelona) for 72 h at 25ºC in Innova 4000 benchtop incubator shaker (New Brunswick Scientific, New York, USA) set at 220 rpm prior to nitrile hydratase induction by transfer into a defined media. The composition of the defined media was as follows: K_2HPO_4 4.97 $g.l^{-1}$, KH_2PO_4 0.05 $g.l^{-1}$, $CaCl_2.2H_2O$ 0.05 $g.l^{-1}$, $FeSO_4.7H_2O$ 0.09 $g.l^{-1}$, $MgSO_4$ 0.02 $g.l^{-1}$ and 1 ml trace element solution, based on that by Layh *et al.*(1997). Glucose (1.8 $g.l^{-1}$) and inducer (30 mM benzamide) addition into the defined media was done after autoclaving. The cells were centrifuged at 27,216 × g, for 20 min and the supernatant was discarded. The cellular pellet was water washed twice prior to it being re-suspended in 100 mM phosphate buffer, pH 7.0 (average wet cells was 1.0 $mg.ml^{-1}$).

Biotransformation

Wet cells (1.0 g) were added to a solution containing 100 mg of the respective substrate (first solubilised in 1 ml methanol) in 100 mM Tris buffer (either at pH 7.0 or at pH 9.0). The resulting mixture was then stirred at 30°C with product generation being monitored by HPLC. The reactions were stopped by centrifugation at 15,344 × g for 15 min with the supernatant being decanted and extracted using three volumes of ethyl acetate. The cellular pellet was also extracted with three volumes of ethyl acetate. The extracted supernatant fractions as well as the cellular pellet extract were combined and then water and brine washed before drying over anhydrous magnesium sulphate. Concentration was performed under reduced pressure with low temperature to minimise thermal decomposition of the samples. Product purification was done by column

chromatography using varied ratios of Hex/ EtOAc for the β-hydroxy nitrile reactions or Hex/ EtOAc as well as MeOH/ DCM solvent systems in case of the β-amino nitriles reactions.

Extract purification of β-hydroxy nitrile reactions were started with a Hex/ EtOAc solvent system (20: 80, %v/v), then increased gradually to a final volume of 100% EtOAc. Nitrile eluted first at lower polarity, followed by amide and the acid. Purification of β-amino nitriles reactions was achieved using a 40 cm flash column, packed with silica gel (silica gel 60) and eluted with a Hex: EtOAc (50: 50, %v/v) solvent mixture, increased gradually up to 100% EtOAc to remove the β-amino nitrile. Once the nitrile was removed the solvent system was changed to MeOH/ DCM (5:95, %v/v) and increased to 20% MeOH to elute the β-amino amide

Cell disruption

Cells were pressure disrupted using the Constant Systems Cell Disruptor (Constant Systems Limited, Northlands, UK) at 20 kPsi. The harvested biomass was re-suspended in potassium phosphate buffer (pH 7.5, 50 mM) to obtain a 30% w/w cell suspension. This solution was subjected to 3 passes in the cell disruptor. The disrupted cells were treated with 0.1 % w/v polyethylenimine to facilitate precipitation of genetic material. This solution was stirred on a magnetic stirrer plate, Heidolph MR 200 (Heidolph, Germany) at 4°C for 1 h. Cell debris was removed by continuous centrifugation (15,000 x g, 10 min) and the enzyme solution concentrated by ultrafiltration through a 10 kDA Prostak ultrafiltration module (Millipore Corporation, USA).

Ammonium sulphate precipitation

Ammonium sulphate (35.2 g) was slowly added to 200ml nitrile hydratase cell free extract solution for 30 min while stirring at 200 rpm on a magnetic stirrer. This solution was stored at 4°C with stirring at 200 rpm to precipitate low molecular proteins, and left overnight in cold room.

The 30% ammonium sulphate precipitated proteins were recovered by centrifugation in a Beckman Coulter Avanti J26 XPI (Beckman Coulter Inc., USA) using JA -14 rotor at 22,095 × g, at 4°C for 30 min, and suspended in 10ml 20 mM Tris-HCl, pH 7.5,buffer. The supernatant (400 ml) obtained after centrifugation was subjected to 70% ammonium sulphate precipitation.

Ammonium sulphate (NH_4SO_4), 58.59 g, was slowly added to 400 ml supernatant to nitrile hydratase solution for 30 min while stirring at 200 rpm. The solution was stored at 4°C with stirring at 200 rpm for 1 h. Nitrile hydratase was recovered by centrifugation as above. The pellets were resuspended in 400 ml 20 mM Tris-HCl, pH 7.5.

2.3.7 Post Precipitation Diafiltration

Pellets from the 70% NH_4SO_4 precipitated pellet were concentrated to 150 ml by filtering through 10 kDa cross-filtration membrane in a Minimate^{TM} TFF system (Pall Corporation, USA), washed with 500 ml 20 mM Tris-HCl, pH 7.5, followed by 500 ml 50 mM sodium chloride in 20 mM Tris-HCl, pH 7.5. The nitrile hydratase active retentate (150 ml) was collected and clarified by anion-exchange.

Anion Exchange

Batch anion exchange was carried out in Q-Sepharose resin (Sigma-Aldrich, USA) eluting washing first with 500 ml 50, 200 and 400 mM NaCl (in 20 mM Tris-HCl, pH 7.5) in a stepwise gradient.

Post Anion Exchange Diafiltration

Nitrile hydratase (800ml) from the anion exchange step was concentrated to 150ml as above and washed with 500ml nanopure water and 20 mM Tris-HCl, pH 7.5 and then concentrated to 65 ml to provide purified nitrile hydratase solution. This enzyme solution was stored at 4°C until use.

2.4 Results and Discussion

2.4.1 Method Development

The RP-HPLC method development was on pure standards to obtain separation between the substrate and biotransformation products. The suitable conditions were chosen after several trials with a mobile phase composition of 0.1% TFA in water, methanol and acetonitrile in various proportions at different flow rates and column temperatures (table 7.1). After optimisation the conditions were adjusted to use a mobile phase composition of 0.1% TFA in water and acetonitrile (70:30 %v/v), at 0.5 ml.min-1 flow rate and column temperature of 25°C were finally selected in order to give optimum separation of analytes, and short analysis run time when using X-terra MS (or RP) C18 3.5 µm column. A typical ultra-violet (UV) chromatogram of a mixture of benzamide, benzoic acid and benzonitrile, as obtained on X-terra MS C18 3.5 µm column, is shown in figure 2.1. Under the optimised chromatographic conditions, benzamide, benzoic acid and benzonitrile peaks were well resolved with retention times (R_t) of 1.18, 1.81, and 2.87 min, respectively.

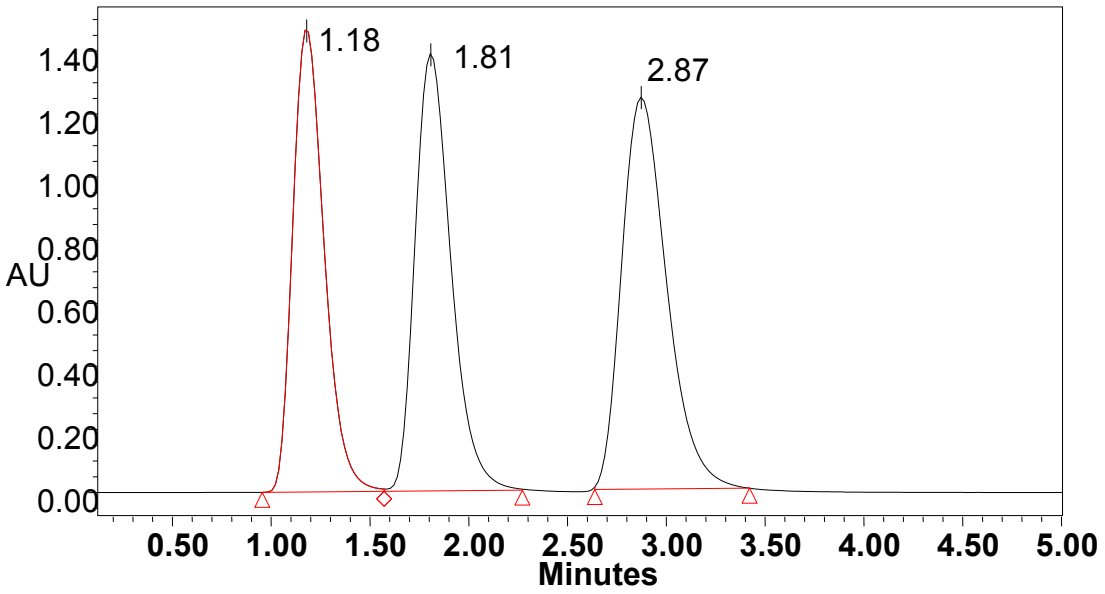


Figure 2.1: A typical UV Chromatogram showing the separation of benzamide (R_t = 1.18), benzoic acid (R_t = 1.81) and benzonitrile (R_t = 2.87)

The experiment was run on the X-terra MS C18 5 µm column; mobile phase0.1% TFA and acetonitrile (70:30 v/v); flow rate of 0.5 ml.min^{-1}, and a column temperature of 25°C.

The Waters X-Terra MS (or RP) C18 3.5µm column, like the monolithic column used by Martínková *et al.* (2008), achieved similar short run times (≤ 3.0 min) of analysis of benzonitrile and the products of its enzymatic conversion.

The figure 2.2 shows that UV absorption spectra of benzamide, benzoic acid and benzonitrile with maxima at 226.4 228.8 and 224.1 nm, respectively. The average detector optimal wavelength was chosen at 225 nm; close to the 228nm opted for by Martínková *et al*. (2008).

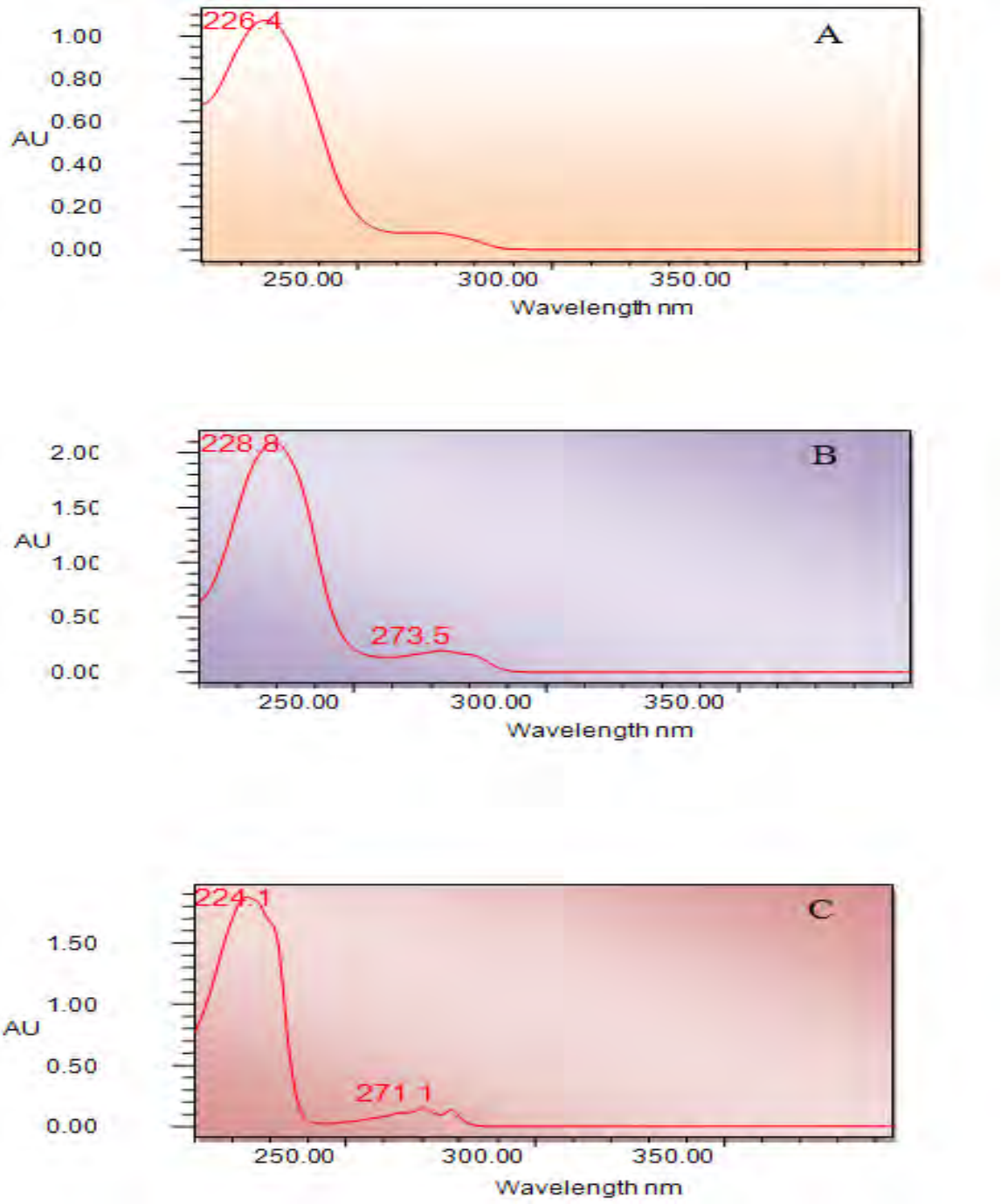


Figure 2.2: UV absorption spectra of (A) - benzamide, (B) - benzoic acid and (C) - benzonitrile.

2.4.2 Method Validation

2.4.2.1 Linearity

The calibration curves (figure 2.3), show a linear relationship between peak areas and concentrations over the concentration range of 0.1 to 3.0mM for the three analytes. The correlation coefficients (R^2) were 0.997, 0.993, and 0.998 for benzamide, benzoic acid and benzonitrile, respectively.

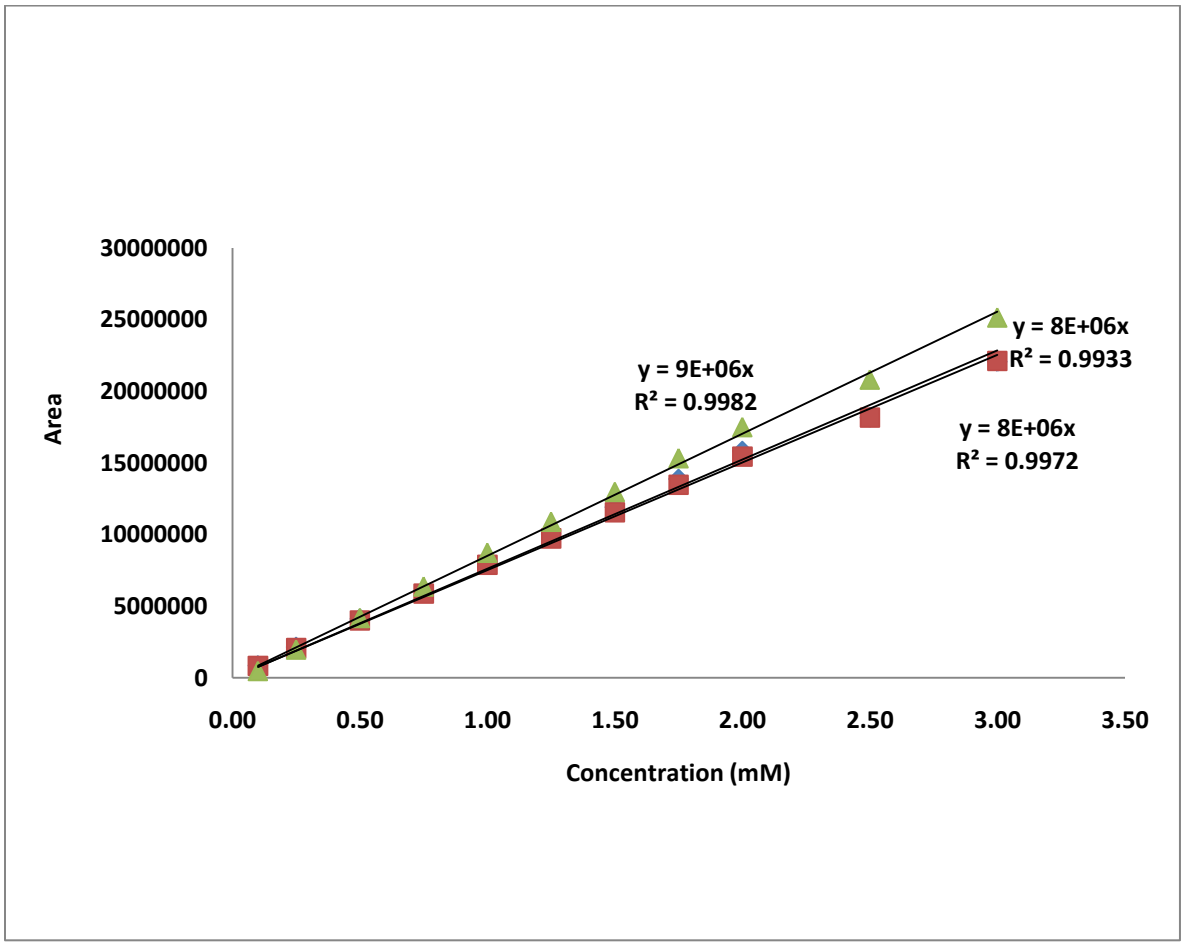


Figure 2.3: Calibration curves of (♦) Benzamide, (■) Benzoic acid and (▲) Benzonitrile standards, eluted at 1.16, 1.85, and 3.02 minutes, respectively.

2.4.2.2 Precision

The results were satisfactory for both intra-day and inter-day analysis with the relative standard deviation being less than 2.2% for the tested compounds (table 2.1), which indicated that the method had good precision.

Table 2.1: Intra – and Inter-day precision results, %RSD = SD/mean × 100 (Miller and Miller, 1993).

		Intra-day[a]			Inter-day[b]		
Compound	Actual amount (mM)	Mean of detected amounts (mM)	Standard Deviation (SD)	%RSD	Mean of detected amounts (mM)	Standard Deviation (SD)	%RSD
Benzamide	2.0	2.15	0.05	2.2	2.1	0.03	1.4
Benzoic Acid	2.0	2.16	0.05	2.1	2.1	0.03	1.4
Benzonitrile	2.0	2.14	0.04	2.0	2.1	0.04	1.9

[a]Analysed on the same day (n=9). [b]Analysed on nine different days (n=9)

2.4.2.3 Accuracy

This RP HPLC method of separating benzonitrile and its related hydrolysis products afford 100.6 to 106.1% recoveries and relative standard deviation with less than 6.5% of the benzonitrile, benzamide and benzoic acid as listed in table 2.2. The over recoveries of 1.00 mM standards might be due to co-elution of impurities. However, from this study it is clear that the results are repeatable within the experimental conditions.

Table 2.2: %Recovery[a] of benzamide, benzoic acid and benzonitrile.

Analytes (mM)		Benzamide	Benzoic Acid	Benzonitrile
1.0	**Average %** RSD%	**106.1** 2.6	**105.6** 0.5	**104.1** 1.7
1.5	**Average %** **RSD%**	102.3 1.1	100.9 1.7	101.4 1.2
2.5	**Average %** **RSD%**	**100.6** 3.5	**102.4** 4.3	**102.2** 6.4

[a] %recovery is defined as the found concentration/ theoretical concentration, expressed as percentage; n = 3

2.4.2.4 Limit of Detection and Quantification

Calculated values of LOD and LOQ from the calibration curve for benzonitrile and its hydrolysis products are shown in table 2.3.

Table 2.3: Limit of quantification and limit of detection data for the analytes.

Analyte	**LOD (mM)**	**LOQ (mM)**
Benzamide	**0.11**	**0.17**
Benzoic Acid	**0.37**	**1.11**
Benzonitrile	**0.03**	**0.10**

Calculated LOD and LOQ of benzoic acid were 0.37 mM and 1.11 mM, respectively. These values are higher than that of benzamide and benzonitrile, which can be attributed to the differences in molar absorptivity of benzoic acid. Benzoic acid has less

molar absorptivity and thus higher detection limits as compared to benzamide and benzonitrile. These higher limits are however still good enough for detecting benzoic acid.

2.4.3 Nitrile Hydratase Partial Purification

The results on the partial purification of nitrile hydratase from *Rhodococcus rhodochorus* BAA-870 cells free extract by a combination of ammonium sulphate, diafiltration and batch anion- exchange chromatography as described in methods are summarised in table 2.4. The ammonium sulphate (30-70%) precipitated enzyme showed 0.6–fold purification when concentrated by diafiltration. After anion-exchange, the enzyme was purified to about 0.4-fold with a specific activity of 351.8 $U.mg^{-1}$ determined using benzonitrile and recovery of 41.6% (table 2.4). The specific activity (351.8 $U.mg^{-1}$) of the partial purified enzyme is comparable to that obtained from *Nitriruptor alkaliphilus*by Van Pelt (2010). Although the enzyme was purified according to visualisation with SDS-PAGE (figure 4.2), activity losses of this multi-subunit enzyme occurred at every step, resulting in lower specific activity and hence purification. The purification did however remove any nitrilase present (mass ~ 42 kDa, Frederick, personal communication) which may have otherwise resulted in confusion over the enzyme responsible for catalysis.

Activity Calculation

Total activity ($U.ml^{-1}$) of the Nhase in 50 mM phosphate buffer (pH 7.5) was calculated as follows:

$U.ml^{-1}$ = $[(mM \times V_T) \times Dil_{(R)}] \div t \times Dil_{(S)} \times 1000 / V_{(S)}$

V_T = reaction total volume (0.4 ml)

$Dil_{(Reaction)}$ = reaction solution dilution factor (4)

T = reaction time (5 minutes)

$Dil_{(Sample)}$ = enzyme solution dilution factor

$V_{(Sample)}$ = enzyme solution actual volume added (0.1 ml)

Table 2.4:Nitrile Hydratase Purification Data

Step	Volume (ml)	Total Units (U)	Activity ($U.ml^{-1}$)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Yield (%)	Purification factor
Cell free extract	200	14694	73.5	15.6	941.9	100	1.0
Supernatant 30% NH_4SO_4	217	12689	58.5	14.1	813.4	86.4	0.9
Pellet 70% NH_4SO_4	400	9813	24.5	11.8	831.6	66.8	0.9
Desalting by Diafiltration	120	8553	71.3	15.2	562.7	58.2	0.6
Anion Exchange	120	6453	53.5	14.3	449.2	43.7	0.5
Concentration and desalting by diafiltration	65	5278	81.2	15.0	351.8	41.6	0.4

The partially purified enzyme showed four bands on SDS-PAGE with atomic masses of about 23, 26, 35 and 50 kDa, observed by comparing with the standard protein makers (figure 2.4). The first two bands, as indicated by the arrows may be the expected molecular weight of α- and β-subunits of nitrile hydratase, respectively, since they are in the range of most reported *Rhodococcus* nitrile hydratase masses (table 2.5). Based on the gene sizes these should be 23 kDa and 26 kDa (Frederick, personal communication). The partially purified *Rhodococcus rhodochrous* BAA-870 nitrile hydratase was subsequently immobilised using the SphereZyme™ enzyme self immobilisation technique as described in Chapter 3.

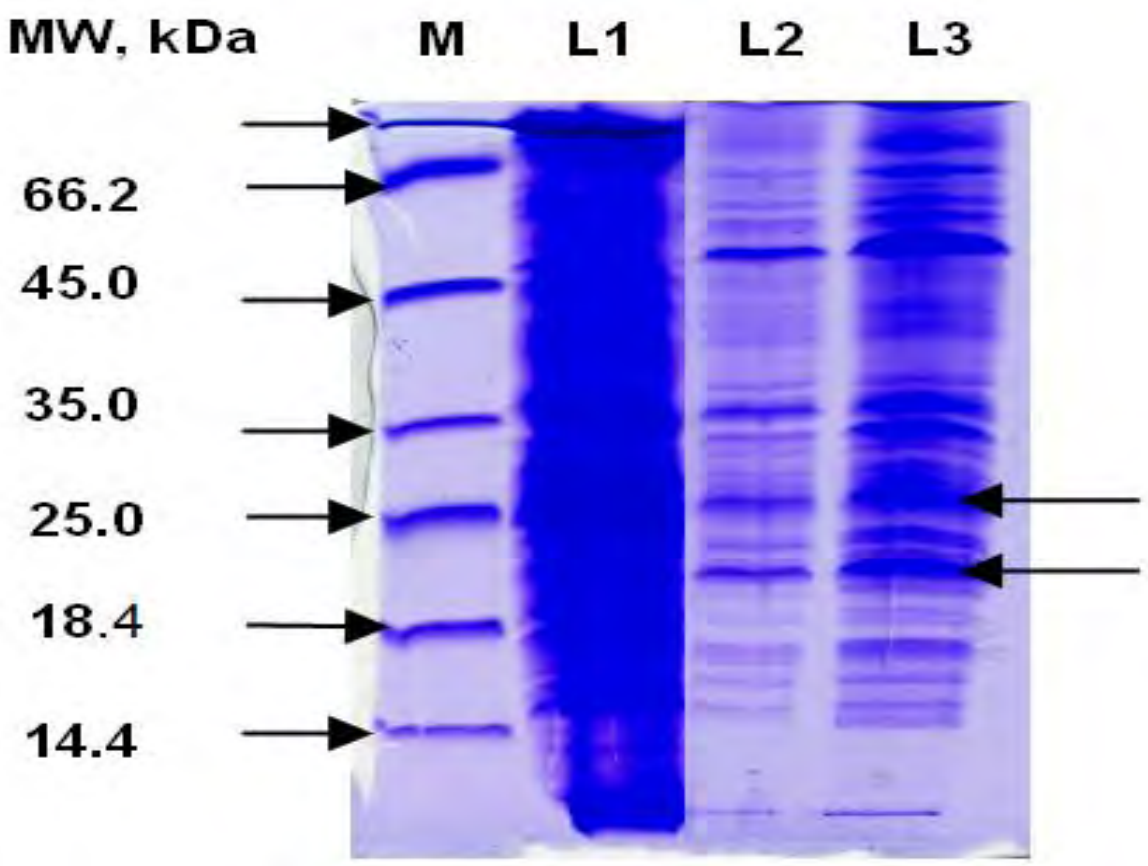


Figure 2.4: 12% SDS-polyacrylamide gel showing anion purification steps.

Lane M standard marker proteins (kDa): Lane 1 cell free extract; lane 2 sample after anion exchange, and 3 concentrated by diafiltration (the direction is from top, cathode, to bottom, anode).

Table 2.5: Molecular masses of some purified Nhases.

Name of organism	Molecular mass (kDa)[a]	Subunits Molecular Mass (kDa)	References
Agrobacterium tumefaciences d3	69 (4)	27.0	Bauer *et al*., 1994
Arthrobacter sp. J1	420 (2)	24.0	Asano *et a*l. 1982
Bacillus RAP c8	(4)	α - 28.0 β – 29.0	Pereira *et al*., 1998
B. pallidus Dac 521	110 (4)	α - 27.0 β – 29.0	Cramp and Cowan, 1999
Nitriliruptor alkaliphilus	19.3	α - 26.0 β – 28.0	Van Pelt, 2010
Rhodococcus sp. N774	70	α - 28.5 β – 29.0	Endo and Watanabe, 1989
Rhodococcus sp. YH3 - 3	130	α – 27.1 β – 34.5	Kato *et al*., 1999
R. equi A4	74	α – 25.0 β – 25.0	Přepechalová *et al*., 2001
R. rhodochorus PA - 34	86	α – 25.0 β – 30.0	Prasad *et al*., 2009
R. rhodochorus	-	α – 23.0 β – 26.0	Present studies

[a] Numerical digit in parenthesis represents the possible numbers of α- and β.

2.5 Conclusion

A simple and efficient reversed-phase HPLC method for separation of benzonitrile and its hydrolysis products with a X-Terra MS (or RP) C18 3.5µm column and eluent consisting of 0.1% TFA (in water) and acetonitrile (70:30, %v/v), at a flow rate of 0.5ml.min^{-1}, and column temperature maintained at 25ºC was found to be accurate, precise, and linear across the studies analytical range. The limit of detection (LOD) for the analytes ranged from 0.03 to 0.37 mM and limit of quantification (LOQ) from 0.10 to 1.11 mM. Elution was within 3.5 minutes, and thus the column can significantly accelerate conversion of the analytes without compromising separation or sensitivity. This method could be applied in routine qualitative and quantitative analysis of the analytes.

The developed reversed HPLC method successfully quantified benzonitrile, benzoic acid and benzamide present in nitrile hydratase assay samples. The specific activity of partially purified nitrile hydratase from *Rhodococcus rhodochrous* ATCC BAA-870 determined was 351.8 U.mg^{-1}.

The α- and β–subunits of partially purified nitrile hydratase from *R. rhodochorus* BAA-870 had molecular weights around 23 and 26 kDa.

Chapter 3: Immobilisation of Nitrile Hydratase

3.1 Introduction

The SphereZymes™ technology is a novel enzyme self-immobilisation strategy whereby enzymes are covalently cross linked in an emulsion (Moolman *et al*., 2005; Brady *et al.,* 2008). Through addition of protein cross-linking agents to a water-in-oil emulsion of an aqueous enzyme solution, this technology allows for the formation of spherical catalytic macro-particles (SphereZymes™) (figure 3.1). SphereZymes™ technology offers several advantages for biocatalysis, such as particle size control and enzyme active site orientation, and in addition it shows improved activity, stability and reusability as compared to known self immobilised enzymes. Although this technology is broadly applicable to variety of enzymes (Moolman *et. al.,* 2005), no nitrile hydrolysing SphereZymes™, are commercially available. Therefore in this study, we intended to immobilise *Rhodococcus rhodochrous BAA-870* nitrile hydratase using the SphereZymes™ technique.

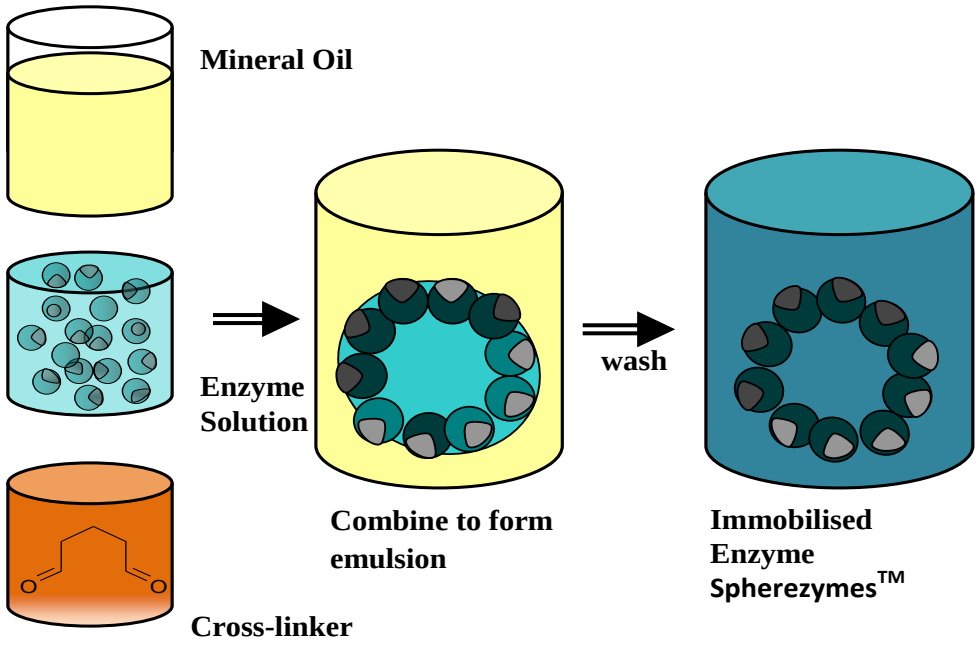


Figure 3.1: Simplified diagram illustrating the formation of the Spherezymes™ particles (adapted from Mathiba *et al.*, 2009).

3.2 Materials and Methods

3.2.1 Materials

Mineral oil and nonoxynol (NP-4) were purchased from Castrol and BASF, respectively. Ammonium sulphate, Tris (Trizma® base, minimum 99.9 % titration), 25% glutaraldehyde, (Glut, grade II), ethylenediamine (EDA), polyethyleneimine (PEI), ethanolamine, bovine serine albumin (BSA), benzonitrile, benzamide and benzoic acid were purchased from Sigma Aldirch Pty (Ltd). Trifluoroacetic acid (TFA), acetic acid, sodium dihydrogen phosphate (NaH_2PO_4), potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4), were all purchased from Merck (Germany). Acetonitrile (ACN), n-heptane, n-isooctane, n-hexadecane (all HPLC grade)

were purchased from LabScan (Poland). Nitrile hydratase from *Rhodococcus rhodocrous* (specific activity of 351.8 $U.mg^{-1}$) was produced at CSIR Biosciences laboratory (Chapter 2). 3-Hydroxy-4-phenoxybuanenitrile (3OH4PBN); 3-hydroxy-3-phenylpropanenitrile (3H4PPN); 4(4-chlorophenyl)-3-hydroxybutanenitrile [4(4ChlP) 3HBN]; and 4-(benzyloxy)-3-hydroxybutanenitrile (4Bz3HBN) were synthesized at CSIR, Biosciences laboratory.

3.2.2 Methods

3.2.2.1 Nitrile hydratase SphereZymes™ formation

Initial cross-linking experiments were performed in microtiter plates (Whitehead Scientific (Pty), Ltd, Brakenfell, SA) under scaled down conditions. Various protein concentrations at different cross-linker concentrations were investigated. Solutions of partial purified nitrile hydratase of 15 $mg.ml^{-1}$ and 0, 5, 10, 15 and 20 $mg.ml^{-1}$ BSA were prepared in 50 mM Tris-HCl buffer (pH 7.5). Samples (50 µl) of each solutions were aliquoted into a microtiter plate, to which different volumes of various cross-linkers (2 µl – 10 µl) were added. The cross-linker solutions were made of 25% glutaraldehyde ("Glut"; grade II), EDA (0.33 M, pH 6), and PEI. The plate was covered and left at room temperature, monitored for the first 30 min, and thereafter hourly for 3 h. All samples that had solidified (indicating successful cross-linking in aqueous solution) were investigated for SphereZymes™ formation in 5 ml mineral oil.

SphereZymes™ were prepared for each of the enzyme solutions with various cross-linker volumes of 10, 20, 30, 40 and 50 µl using the standard SphereZymes™ preparation method according to Brady *et al.* (2008), with some modifications. The surfactant, nonoxynol (50 µl) was dissolved in 5.0 ml of mineral oil with magnetic stirring at 4ºC for 5 min. The aqueous phase was prepared by adding 50 µl 100 mM of the active site protectant solution (3-hydroxy-3-phenylpropanenitrile) to 100 µl enzyme preparation. This solution was allowed to react for 5 min, allowing the substrate to saturate the active sites (but for too short a period to allow for significant conversion of the substrate). Concurrently, in a separate vessel the cross-linking agent was prepared

by reacting Glut (25 % aqueous solution grade II) with 5% PEI in a 1:1 ratio for 10 min at 25ºC. Both the active site protectant reaction and the cross-linker reaction were completed at the same time. The cross-linking agent was then added to the enzyme solution, mixed thoroughly and immediately emulsified in the oil phase with magnetic stirring for 1 min at 300 rpm (4°C). This emulsion was stored at 4ºC with mixing to prevent the enzyme droplets from settling during cross-linking, and then left overnight at 4°C, with mixing in a roller drum, Model TC-7 (New Bruswick Scientific, USA) for SphereZyme™ formation. The immobilised enzyme preparation was recovered by centrifugation in an Allegra™ X-22 R centrifuge (Beckman Coulter, Inc, USA) fitted with swing bucket rotor 3,000 ×*g* for 20 min. The spheres were washed three times with 30 ml of 50 mM Tris-HCl, pH 7.5, containing 1.mM ethanolamine, and once with 30 ml of 50 mM Tris-HCl, pH 7.5, for excess glutaraldehyde quenching. The spheres were resuspended to 2 ml in 50 mM phosphate buffer, pH 7.5. A sample of manufactured SphereZymes™ was retained for activity maintenance investigations.

3.2.2.2 Assay of Nitrile Hydratase activity

Nitrile Hydratase solution (100µl) was added to a 2.0 ml Eppendorf tube containing 300 µl of 10 mM benzonitrile (in minimum methanol, MeOH) dissolved in 50mM K_2HPO_4/ KH_2PO_4 buffer (pH 7.5). The total assay volume was 400 µl. Although most nitrilases are relatively stable to temperatures up to 55°C for short periods, the reaction mixture was incubated at a lower temperature, i.e. 30ºC, in an TS100 thermo shaker (BOECO, Harmaburg, Germany) shaking at 1,000 rpm for 5 min, due to the instability of most nitrile hydratases at high temperatures. The reaction was stopped by addition of 1,200 µl of a solution mixture of 0.1% trifluoroacetic acid and acetonitrile (HPLC grade), 70:30 v/v. All tubes were then centrifuged at 13,000 rpm (Biofuge Pico Microfuge, Heraeus Instruments, USA) for 10 min. The resulting aliquot of each sample was transferred into a 2 ml HPLC vial and analysed for nitrile hydratase activity in an HPLC system. Each sample was analysed in triplicate.

3.2.2.3 Activity of nitrile hydratase Spherezymes™ in the presence of organic solvents

An exact amount of 5 mg wet sample of 1.20 U.mg^{-1} nitrile hydratase Spherezymes™ (NHSZ) was weighed into a 2 ml eppendorf tube and dissolved with either 500 µl of 50 mM phosphate buffer, pH 7.5 or co-solvents containing 10, 25, 50 and 75% (v/v) n-heptane, isooctane or n-hexadecane saturated with 50 mM phosphate buffer, pH 7.5. An amount of 100µl was taken at 0 and 60 min for activity assay.

In the case of Nhase, 85µl of Nhase (59 mg.ml^{-1} protein) was pipetted into a 2.0 ml eppendorf and mixed with 415 µl of solvents as above.

3.2.2.4 Protein Analysis

Protein concentrations were measured by the Bradford method (Bradford, 1976) using the Bio-Rad-Coomassie® Protein Dye Reagent according to the manufacturer‟s protocol (BioRad, USA). Dye reagent was prepared by diluting 1 part dye reagent with 4 parts MilliQ water. The reagent was filtered through Whatman® number 1 to remove dye particulates as per the manufacturers‟ specification.

Bovine Serum Albumin (BSA) was used as a reference standard. Dilutions of BSA protein standards in the range 0.05 to 0.5mg.ml^{-1}were prepared and assayed to generate a standard curve according to the manufacturer‟s protocol. Samples of 10 µl protein standards or protein sample were mixed with 200 µl diluted dye reagent in a flat-bottomed microtitre plate. The plate was incubated at room temperature for at least 5 min and amount of protein measured using spectrophotometric analysis at 595 nm in a PowerWave™ HT microtiter plate reader (BioTek® Instruments Inc., USA). Protein concentration was determined by linear regression analysis of the standard curve of optical density (OD) at 595 nm. All assays were performed in triplicate.

3.3 Results and Discussion

3.3.1 SphereZymes™ Formation

Cross-linking was visualised as the formation of solid mass or precipitate and an orange-brown colour formation after 3 h as reported by Jordaan *et al*. (2009b), although their time was 24 h.

The initial microtiter plate scale down investigation was successful for protein volume of 50 µl at various protein concentrations with cross-linkers glutaraldehyde, glutaraldehyde: 5% PEI (w/v, pH 11), 1:1 v/v, at volumes of 2 µl to 10 µl. No particles were formed when Glut: EDA was used as a cross linking agent for nitrile hydratase SphereZyme™ formation, even when the protein was supplemented with albumin as a proteic feeder.

A 25% v/v glutaraldehyde (grade II) was used, since it is generally the cross-linker of choice as it is inexpensive and readily available commercially (Sheldon, 2007). Cross-linking occurs through reaction of the primary amine groups of lysine, abundant on the enzyme surface, which form a Schiff base with aldehyde groups of the cross linking (figure 3.2). Cross-linking was observed in the initial cross-linking experiment with glutaraldehyde.

Aqueous alkaline polyethyleneimine (PEI) can be used to increase the length of glutaraldehyde and has been shown to improve the cross-linking efficiency of proteins (López-Gallego *et al*., 2007). Polyethyleneimine contains two terminal primary amines which react with terminal aldehyde groups of glutaraldehyde. The elongation of the glutaraldehyde using PEI could potentially provide improved substrate diffusion and thereby improved reactivity between the cross-linked enzyme and the substrate (Jezkova *et al*., 1997; He *et al*., 2010).

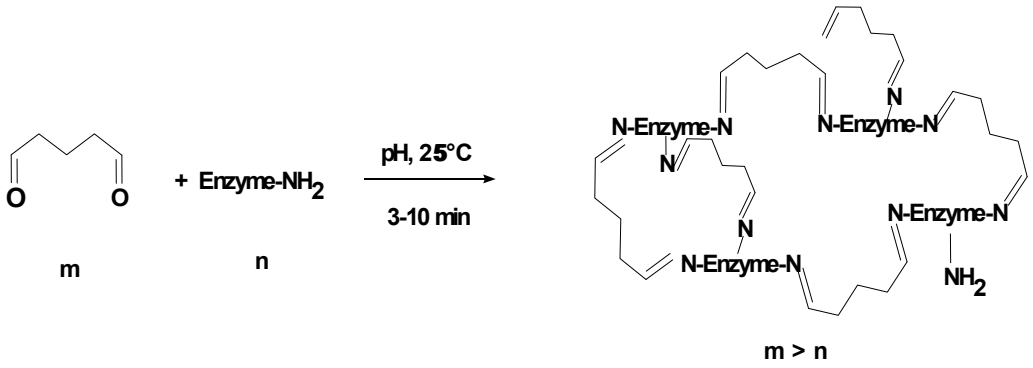


Figure 3.2: Proposed method for enzyme cross linking using a bi-functional cross-linking agent (after Cao, 2005b).

PEI is more nucleophilic at an alkaline pH and thereby more reactive toward electrophiles such as the carbonyls of aldehyde groups (Boussif *et al*., 1995). Thus it was able to cross-link with the enzyme nitrile hydratase forming nitrile hydratase Spherezymes™ (NHSZ).

Use of glutaraldehyde and EDA mixture as a cross-linker has been shown to improve the cross-linking efficiency of proteins (Cao and Elzinga, 2003). The elongation of the glutaraldehyde using EDA occurs because of the reaction of ethylenediamine‟s two terminal primary amines with terminal aldehyde groups of glutaraldehyde. Lack of precipitation formation when 0.33 M EDA and 25% v/v glutaraldehyde was used as cross-linker might be due to the following factors: short period of cross-linking time, lower volume of cross-linker mix, lower concentration of nitrile hydratase stock solution and EDA solution.

Based on the microtiter plate results, nitrile hydratase SphereZymes^{TM} were prepared at 5 ml mineral oil scale with 100 µl nitrile hydratase solution (15 mg.ml^{-1}) enhanced with

BSA (5 – 20 mg.ml^{-1}) and various cross linker volumes of 10 to 50 µl using SphereZymes^{TM} procedure as described in section 3.3.1.

From the results obtained, activity was not maintained when using glutaraldehyde as a lone cross-linker. This might be due to the introduction of more -NH groups (from the enzyme) to react with free -OH groups of glutaraldehyde to cross-link, resulting in aggregation of individual SZs to each other hence decreasing substrate accessibility to the active site of SZs, or deactivation of enzyme resulting from active site penetration by glutaraldehyde (Chae *et al*., 1998).

SphereZymes^{TM} were successfully manufactured at all the cross-linker glutaraldehyde: PEI (1:1 v/v) volumes (figure 3.3), thereby confirming the validity of the initial scaled down microtiter plate investigation. The SphereZymes^{TM} had high activities at low volumes of the cross-linker, with 20 µl cross-linker being the optimum (figure 3.4).

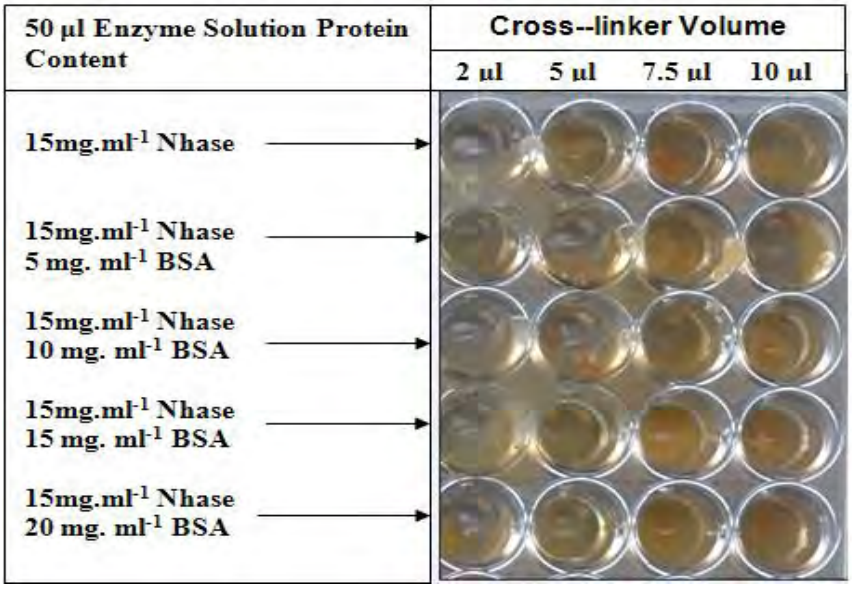


Figure 3.3*:* Example of wells showing *c*ross-linking efficiency of nitrile hydratase with cross-linker glutaraldehyde: PEI (1:1 v/v) after 3 h.

The formations of an orange-brown precipitate as shown in the wells indicate the suitability for cross linking of nitrile hydratase.

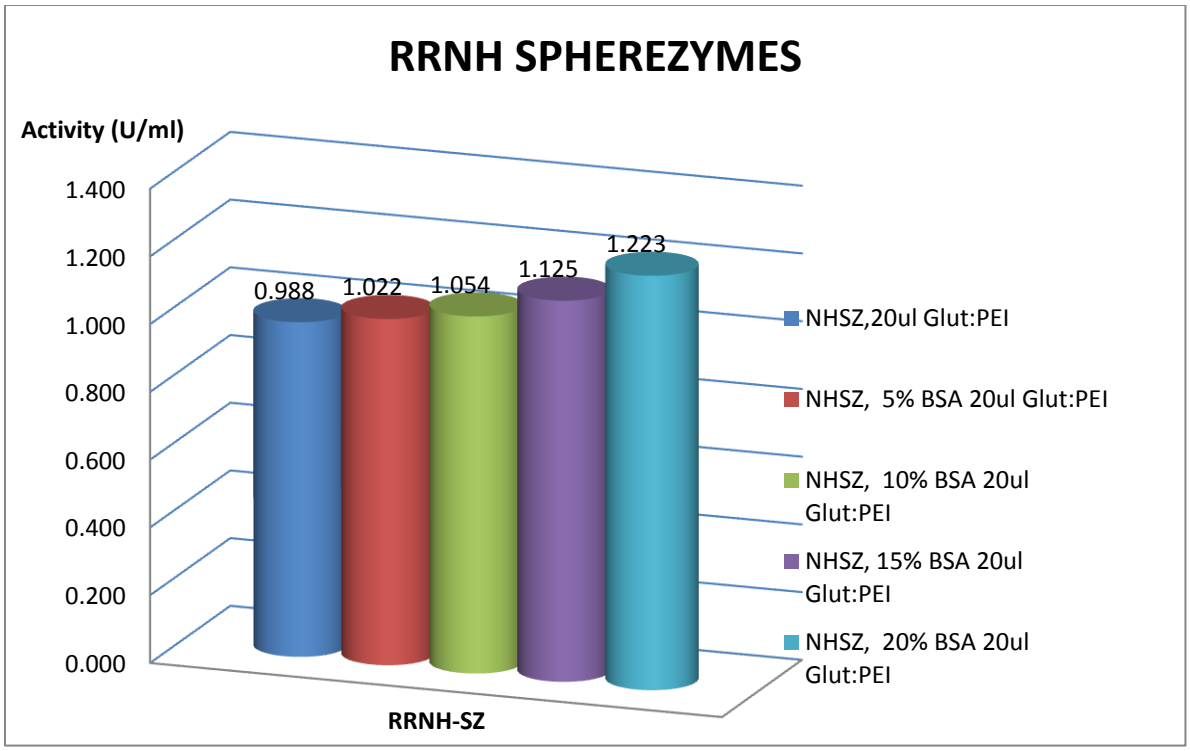


Figure 3.4: Scale-up cross-linking of nitrile hydratase Spherezymes™ at 100 µl of 15 mg.ml^{-1} enzymes enhanced with various quantities of BSA (5 to 20 mg.ml^{-1}).

3.3.2 Protein Concentration

The concentration of protein was correlated to the formation and activity of SphereZymes™ produced. The maximum activity maintenance of nitrile hydratase SphereZymes™ towards benzonitrile was achieved for 35 mg.ml^{-1} total protein concentration with 20 µl cross-linker (see section 3.3.1). Although the SphereZymes™ had lower specific activity above 50 mg.ml^{-1}, the results indicated that the specific activity of the SphereZymes™ remains relatively constant at high protein concentrations.

3.3.3 Protectants

A protectant is defined as a compound that protects the enzyme active site during cross linking, and it"s an important component of SphereZymes™ preparation as it has been shown to increase the activity maintenance for the immobilised enzyme (Brady *et al*.,

2008). The aryloxy and aryl nitriles, 3OH4PBN; 3OH3PPN; 4(4ChlP)3HBN; and 4Bz3HBN that were converted to the corresponding carboxyamides and carboxyacids by *Rhodococcus rhodochrous* ATCC BAA 870 (see chapter 4) were tested for nitrile hydratase active site protection. Although these nitriles can be converted to their corresponding carboxylic acids and amides, the rate of reaction may be slow due to their bulkiness (benzene ring), and thereby allowing cross-linking to take place.

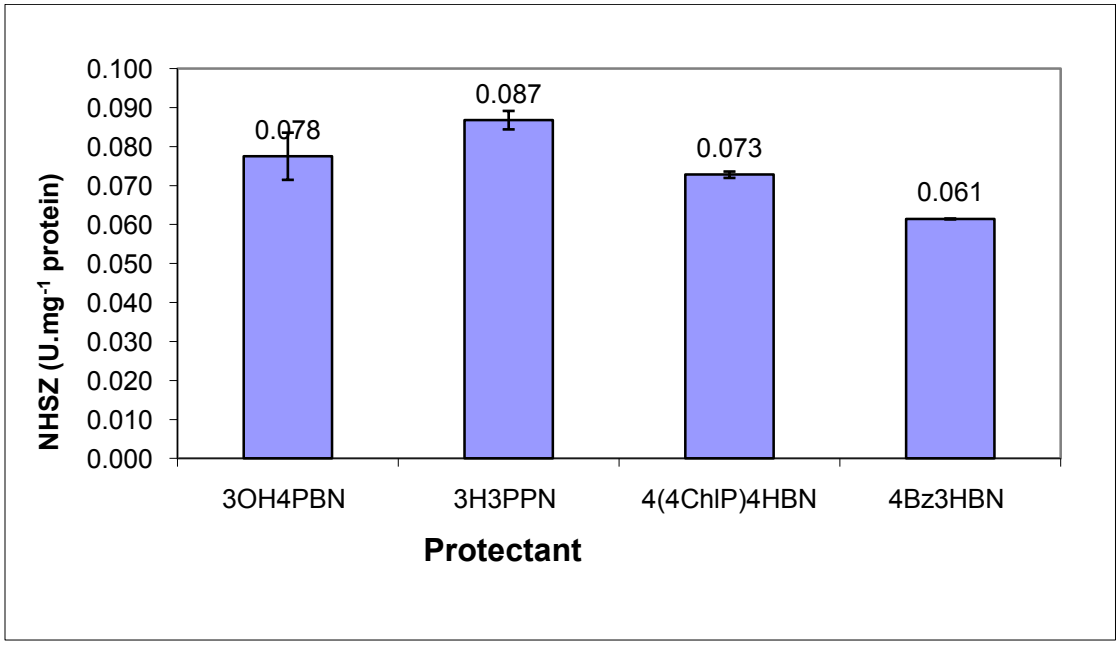


Figure 3.5: Comparison of various protectants on nitrile hydratase activity and activity recovery for NHSZ formation.

All four nitriles showed protectant abilities towards Nhase (figure 3.5) and nitrile, 3-hydroxy-3-phenylpropanenitrile was used as protectant throughout study because of its availability.

3.3.4 The effect of buffer pH on enzyme activity

The effect of pH on activity against benzonitrile was tested using Universal buffer (50.mM acetic acid, 50 mM NaH_2PO_4, 50 mM Tris) at pH ranging from 3.0 to 9.0. The results showed that both NHSZ and Nhase exhibited pH optimum at 6.0 (figure 3.6),

which compares favourable with other Nhases optima reported in the literature (Martínková *et al.*, 2009). It was observed that the immobilisation process, SphereZymes™, to some extend protected Nhase in the acidic range (from pH 3.0 to 6.0). Similar behaviour has been observed with nitrile hydratase CLEA prepared from *Nitriliruptor alkinaliphilus* (Van Pelt, 2010), although the reported pH range is between 4 and 11.

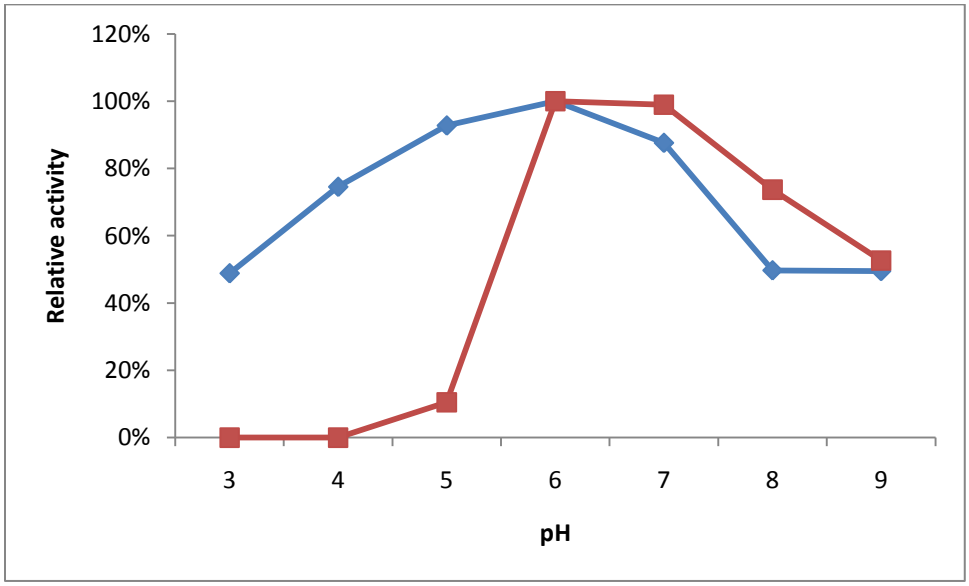


Figure 3.6: Effect of buffer pH on nitrile hydratase activity in free enzyme and SphereZymes™.

The reactions were conducted for 5 min at 30°C in the Universal buffers (50 mM acetic acid, 50 mM NaH_2PO_4, 50 mM Tris) at pH ranging from 3 to 9. (♦) Nitrile hydratase spherezymes and (■) free enzyme (Fredericks's data).

3.3.5 Effect of temperature on enzyme activity

The reactions of the effect of temperature on the activity of Nhase and NHSZ were carried out at various temperatures for 5 min. The temperature optima of both Nhase and NHSZ are similar (30ºC), at pH 7.5 of the reaction medium (figure 3.7). It was observed that the NHSZ had higher relative activity than the Nhase up to approximately 70°C, even though relative activities of both biocatalysts decreased as the temperature

increase. This observation is in agreement with the reported behaviour of immobilised Nhase enzymes in the literature (Chiyanzu *et al.*, 2010; Van Pelt, 2010).

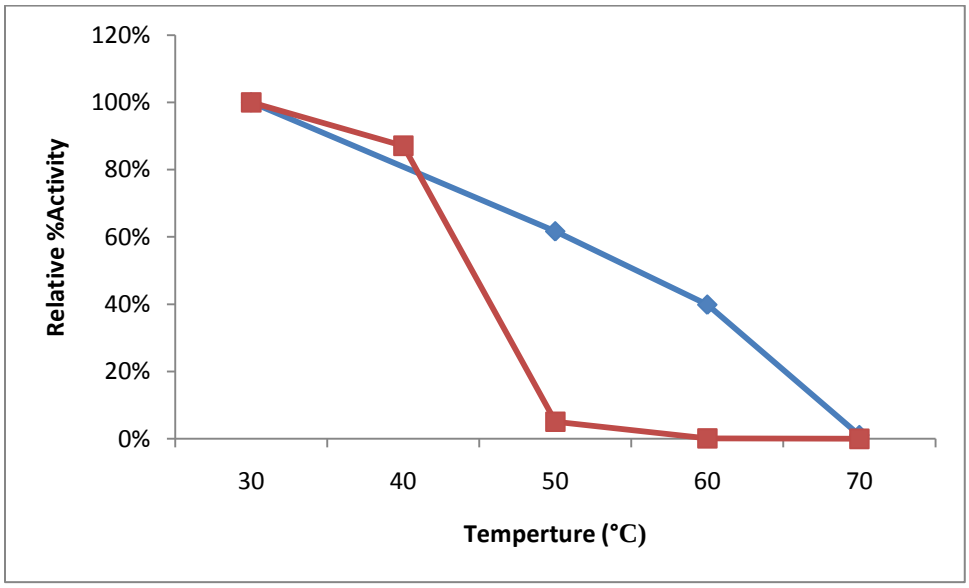


Figure 3.7: Comparison of the temperature activity profiles of free nitrile hydratase (♦) and nitrile hydratase SphereZymes™ (■) in 50 mM potassium phosphate buffer at pH 7.5.

3.3.6 Recyclability

Figure 3.8 shows the activities of same NHSZ batch that was repeatedly washed with 50 mM TRIS-HCl buffer, pH 7.5. NHSZ activity decreased by 66% with every 3x repeated washing. The observation is contrary to that of Chiyanzu *et al.* (2010). In their case, there was about 10% loss of activity after eight cylces.

The continuous loss of activity could be attributed to the following factors:-

- conformational changes; because most enzymes are very sensitive to environmental changes, treating them would unavoidably make them denature (Hou *et al.,* 2007),
- washout of iron or cobalt co-factor , since no metal ions were included in the buffer,

- instability, resulting from disassembly of cross-linking due to repeated washing and centrifugation.

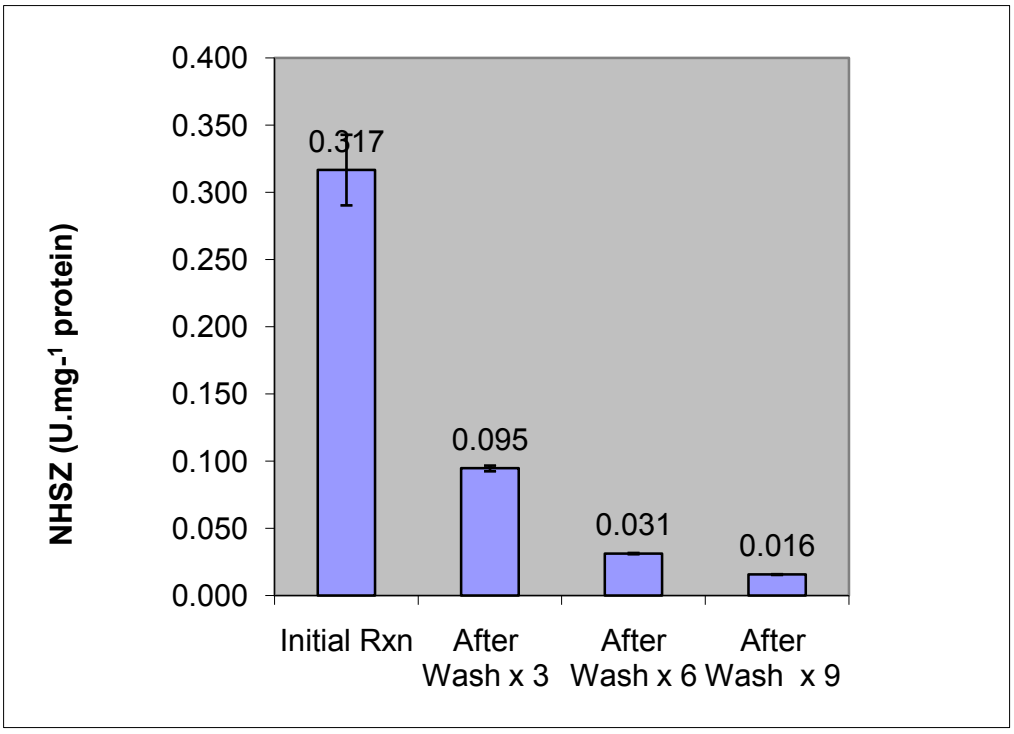


Figure 3.8: Activity of nitrile hydratase SphereZymes^{TM} (15 mg.ml^{-1} Nhase and 20 mg.ml^{-1} BSA) activity through repeated washings and recycles.

No metal ions were included in the buffer.

3.3.7 Activity on Nitrile Hydratase SphereZymes^{TM} (NHSZ) in non-polar organic solvents

The results in table 3.1 show that both NHSZ and Nhase hydrolysed benzonitrile in the presence of non-polar compounds isooctane and n-hexadecane, but the activities were generally lower as compared to control, no co-solvent (50 mM K_2HPO_4 / KH_2PO_4 buffer, pH 7.5). In a case of Nhase, no significant loss of activity occurred in the presences isooctane and n-hexadecane, activities remained high at levels above 6.25% (v/v), but significant losses occur in case of n-heptane (table 3.1). The activity of NHSZ in 2.5%

(v/v) isooctane (127%) is almost similar to that obtained using Nhase from *Rhodococcrus equi* A4 (124%) (Přepechalová *et al.,* 2001), although in their case the co-solvent was 5% (v/v) isooctane. In the case of Nhase, activities are similar to those obtained using Nhase from *Rhodococcrus* sp. DSM 11397 (isooctane, 97%; hexadecane, 105%) (Layh and Willets, 1998). Preference of these two organic solvents by NHSZ may be attributed to solvent hydrophobicity, which shields the NHSZ from the harsh aqueous phase environment (Přepechalová *et al.,* 2001).

It remains to be investigated if the differences in activities are due to enzyme source or structure.

Table 3.1: The effect of n-heptane, isooctane and n-hexadecane on the activities of NHSZ and Nhase towards benzonitrile.

Co solvent	Nitrile hydratase activity retained over 60 min [n]	
	Free enzyme	Immobilised
no co-solvent	159%	116%
n-Heptane 2.5%	11%	0%
n-Heptane 6.25%	0%	0%
n-Heptane 12.5%	0%	0%
n-Heptane 18.75%	0%	0%
Isooctane 2.5%	98%	127%
Isooctane 6.25%	97%	35%
Isooctane 12.5%	91%	15%
Isooctane 18.75%	62%	13%
n-Hexadecane 2.5%	97%	107%
n-Hexadecane 6.25%	102%	137%
n-Hexadecane 12.5%	98%	112%
n-Hexadecane 18.75%	101%	89%

n=3 (each experiment was done in triplicate)

3.4 Conclusion

Nitrile hydratase with specific activity of 351.8 $U.mg^{-1}$ was used to immobilise and manufacture nitrile hydratase SphereZymes^{TM} with specific activity of 1.2 $U.mg^{-1}$. Ingredients of manufactured nitrile hydratase SphereZymes^{TM} were: 5 ml mineral oil, 50 µl NP-4, 100 µl nitrile hydratase (50 $mg.ml^{-1}$), 50 µl 100 mM 3-hydroxy-3-phenylpropanenitrile, and 20 µl 25% Glut: 5% PEI (1:1).

The pH and temperature optima of nitrile hydratase SphereZymes^{TM} manufactured under the experimental conditions are 6 and 30ºC, respectively, and their stabilities are broader than that of free enzyme. It is apparent from the high activity loss results of NHSZ during each 3x washing step that the prepared NHSZ geometry was not stable, thus may not be reused.

Although low specific activity of immobilised partially purified nitrile hydratase of *R. rhodochrous ATCC BAA-870* was obtained as compared to free nitrile hydratase in the presence of organic solvents n-heptane, isooctane and n-hexadecane, its ability to retain activity in the presence of isooctane and n-hexadecane saturated with 50mM K_2HPO_4/ KH_2PO_4buffer (pH 7.5) was demonstrated**.**

Chapter 4: Quantification of the β-Substituted Nitriles

4.1 Introduction

4.1.1 Industrial application of β-amino acids as well β-hydroxy acids

β-amino and hydroxynitriles have been explored as potential sources of β-amino and hydroxy acids, respectively, because these acids are key components of pharmaceutical compounds. β-Hydroxy functional groups occur in β-adrenergic drugs or β-blockers (Wünsche *et al.,* 1996; Kamal and Khanna, 2001; Kamal *et al.,* 2002; Preiml *et al*., 2004; Kamal *et al.,* 2005a; Kamal *et al.,* 2005b; Hammond *et al.,* 2007). These include drugs used for the treatment of cardiovascular disorders such as hypertension, cardiac arrhythmia, ischemic heart disease, antifungal, antibiotic, cytotoxic, antidiabetic and psychiatric disorders. Each of these drugs possesses at least one chiral centre, and an inherent high degree of enantioselectivity in binding to the β-adrenergic receptor.

Presently, a majority of commercially available β-adrenergic blocking agents are marketed as racemates (Bhushan and Thiongo, 1998; Kamal *et al.*, 2005b; Nguyen *et al.*, 2006), due to the difficulty of separation, or the lack of serious known side-effects attributed to the wrong enantiomer. Nevertheless, to avoid the possible undesirable and possibly long term effects of a chiral drug, it is imperative that only pure, therapeutically active forms are prepared and marketed. Hence there is a great need to develop the technology for analysis and separation of racemic drugs.

Much research has also been done into the application of the β-amino acid containing compounds in human therapeutic studies or their use as leads into the discovery of new classes of compounds with particular receptor-binding abilities (Steinhuebel *et al.*, 2009; Imbriglio *et al.*, 2009). Typically, β-amino acids have the additional benefit of exhibiting more stable and predictable secondary structures with peptidases unable to hydrolyse on these peptide bonds. β-amino acid-containing therapeutics are therefore active and

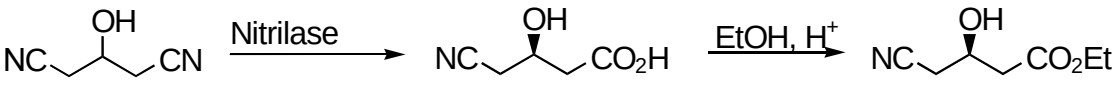
survive cellular degradation longer, thus they can be used in the synthesis of peptidomimetics. A peptidomimetic, in order to function optimally, must reach its target site intact, which is the case for peptides containing β-amino acids. This is largely due to the fact that human peptidases do not recognise β-amino acids as substrates for cleavage, thus extending the in vivo stability of the compound (Brandt *et al*., 2006).

Many of the most promising enantiopure β-amino acids that could be used for peptidomimetic building blocks are, however, not generally available (Steer *et al.*, 2002). Recently enzymatic methods have been established for preparation of enantiopure β-amino acids. For example, *Rhodococcus erythropolis* NCIMB 11540, *Rhodococcus equi* A4 and *Rhodococcus erythropolis* strains have been applied to catalyse the hydrolysis of *N*-protected β-amino nitriles (Chen *et al.*, 2001; Winkler *et al.*, 2005). The enantioselectivity of conversion ranged from 0 to 99% for some nitriles. The *trans*-five membered ring nitriles resulted in amides with enantioselectivity of 94-95% whereas the *trans*-six membered ring nitriles afforded acids with enantiomeric excess (ee) of 87-99%.

There are few reports on the asymmetric hydrolysis of β-hydroxy nitriles using microbial or enzymatic transformations, apart from that of 3-substituted glutaronitrile derivatives (Kakeya *et al.,* 1991; Crosby *et al.,* 1992; Maddrell *et al.* ,1996). Wu and Li (2003) reported the hydrolysis of 3-hydroxy-4-aryloxybutanenitriles using *Rhodococcus* sp. CGMCC 0497, demonstrating that β-hydroxy nitriles could be hydrolysed to their corresponding amides and acids. However, unlike the hydrolysis of α-hydroxy nitriles, they did not obtain an excellent ee for hydrolysis of β-hydroxy nitriles (Wu and Li, 2003; Gao *et al*., 2006).

Diversa Corporation (San Diego, USA) has shown that β-hydroxy nitriles such as the prochiral 3-hydroxyglutaronitrile **(i)** can be desymmetrised, thus generating non-chiral centre, using a nitrilase (figure 4.1) (Robertson *et al*., 2004). Subsequent esterification of the resultant 4-cyano-3-hydroxybutanoic acid **(ii)** affords an intermediate, ethyl-4-

cyano-3-hydroxybutanoate **(iii)** that is used in the manufacture of Lipitor, which is one of the classes of cholesterol lowering drugs.



i **ii** **iii**

Figure 4.1: Selective mono-hydrolysis of 3-hydroxypentanedinitrile (i) by nitrilase to 4-cyano-3-hydroxybutanoic acid (ii) and further esterification to ethyl-4-cyano-3-hydroxybutanoate (iii).

R. rhodochrous ATCC BAA-870, isolated from soil, possesses a nitrile hydratase/ amidase biocatalytic system which is capable of metabolising a wide range of aliphatic and aromatic nitriles and amides as shown by Brady *et al*., 2004. *Rhodococcal* biocatalysts; expressing nitrilases, nitrile hydratases and amidase, are frequently used as whole cell systems or as their component isolated enzymes (Winkler *et. al*., 2005). In this study the biocatalytic asymmetric hydrolysis of β-substituted nitriles to corresponding β-substituted amides and acids by whole cells of *Rhodococcus rhodochrous* ATCC BAA-870 will be studied, and the non-chiral and chiral separation of these hydrolytic products demonstrated by HPLC. Whole cells were used in place of immobilised nitrile hydratase since they are less laborious and were readily available as an alternative.

4.1.2 Polysaccharide-based stationary phases for chiral HPLC

Chiral stationary phases containing amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak® AD-H), or cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel® OD-H) have been widely used for the separation of the enzymatic hydrolysis of nitriles (Kamal and Khanna, 2001; Winkler and Klempier, 2009), while cellulose tribenzoate (Chiralcel® OB-H) has rarely been used. These chiral columns are polysaccharide chiral stationary

phases (CSP) derived from cellulose and amylose polymers. The cellulose derivative contains β-glycoside linkage and forms a linear structure, while the amylose derivative CSP contains α-glycoside linkage and adopts a helical structure. The chiral recognition and resolving ability of these columns depends on polysaccharide linkage and the substituents introduced on the aromatic moieties of the chiral stationary phase. These substituents can interact with the different racemates through hydrogen bonding, dipole interactions, π-π intersections and steric interactions in order to achieve chiral discrimination (Chiral Technologies, 2003).

Chiralcel AD-H® and Chiralpak®OD-H and OB-H columns operate mainly in the normal phase mode. The typical mobile phases used in these columns are based on the non-polar solvents n-hexane and n-heptane, and a modifier such as isopropanol (IPA), methanol (MeOH), ethanol (EtOH) or acetonitrile (ACN). The choice of modifier has dramatic effects on the chiral separation and can also control retention of analytes. Acidic modifiers such as acetic acid and trifluoroacetic acid are also often used to improve separation.

4.1.3 Chiral crown ether-based CSP

Chiral crown ether-based CSPs are synthetic macrocyclic polyethers with the appearance of a crown-like cavity of specific size. The chirality in the crown ether is developed by introducing crown ether oxygens which act as electron donor ligands inside the wall of the cavity (Cavazzini *et al.* 2011).

Crownpak CR(+) is capable of resolving amino acids and other compounds bearing a primary amino group near the chiral centre, and should be operated with aqueous acidic mobile phase to form an ammonium ion. Hence the separation of amides such as sulfoamides and benzamides is not possible on these CSPs (Winkler and Klempier, 2009). Use of perchloric acid ($HClO_4$) is recommended for acids resolution because of its better resolution ability and low UV–absorption. Besides $HClO_4$, phosphoric acid, sulphuric acid, trifluoroacetic acid, nitric acid, hydrochloric acid, methanesulfonic acid

and citric acid can be used in mobile phase. The suitable working pH range of the mobile phase is from 1 to 2.

Recent application of the crown ether based Crownpak CR(+) has been demonstrated by Winkler and Klempier (2009) who successfully resolved unprotected β-amino acids derivates.

In this chapter, non-chiral and chiral analytical methods will be developed and used to quantify compounds present in β-amino nitrile biocatalysis reactions.

4.2 Methods and Materials

4.2.1 Microbial cell cultivation

Strain *Rhodococcus rhodochrous* ATCC BAA-870 was first cultured in a rich media, tryptone soya broth (TSB) obtained from Anatech (Schatlau Chemie, S.A. Barcelona) for 72 h at 25ºC in Innova 4000 benchtop incubator shaker (New Brunswick Scientific, New York, USA) set at 220 rpm prior to nitrile hydratase induction by transfer into a defined media. The composition of the defined media was as follows: K_2HPO_4 4.97 $g.l^{-1}$, KH_2PO_4 0.05 $g.l^{-1}$, $CaCl_2.2H_2O$ 0.05 $g.l^{-1}$, $FeSO_4.7H_2O$ 0.09 $g.l^{-1}$, $MgSO_4$ 0.02 $g.l^{-1}$ and 1 ml trace element solution, based on that by Layh *et al*. (1997). Glucose (1.8 $g.l^{-1}$) and inducer (30 mM benzamide) addition into the defined media was done after autoclaving. The cells were centrifuged at 27,216 × g for 20 min and the supernatant was discarded. The cellular pellet was water washed twice prior to it being re-suspended in 100 mM phosphate buffer, pH 7.0 (average wet cell weight was 0.9-1.0 $mg.ml^{-1}$).

4.2.2 Routine cell maintenance of ATCC BAA 870

R. rhodochrous ATCC BAA-870 cells were cultured onto TSB. The media consists of the following: 1.7% (w/v) casein peptone, 0.3% (w/v) soya peptone, 0.5% (w/v) NaCl, 0.25% (w/v) di-potassium phosphate, and 0.25% (w/v) dextrose (pH approximated at 7).

Cells were cultured overnight at 25ºC in Innova 4000 benchtop incubator shaker set at 220 revolutions per min (rpm) prior to cryopreservation using an equal volume of 40% glycerol in phosphate buffer (100 mM, pH 7). The cryopreserved cells were then stored in an ultrafreezer at -85ºC.

4.2.3 Nitrile hydratase and amidase assay validation

RP-HPLC method development and assay validation was initially determined on benzonitrile, benzamide and benzoic acid (sub-section 2.4.1 and 2.4.2, chapter 2).

4.2.4 Substrate selection for activity determination

Substrates such as 3-amino-3-phenylpropanenitrile, 3-hydroxy-3-phenylpropanenitrile, 3-amino-4-phenylbutanenitrile as well as 3-hydroxy-4-phenoxybutanenitrile, including their various functional analogs, were synthesised inhouse, at CSIR Biosciences laboratory. Thereafter they were hydrolysed as shown in figure 4.2 and figure 4.3.

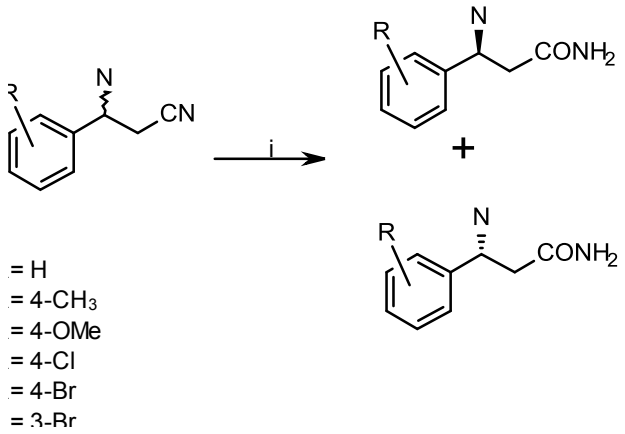


Figure 4.2: A general schematic demonstrating biocatalytic synthesis of the β-amino amides by *R. rhodochrous* sp. (i).

Reaction solvent contain MeOH in Tris buffer (1: 10 v/v), at 30ºC.

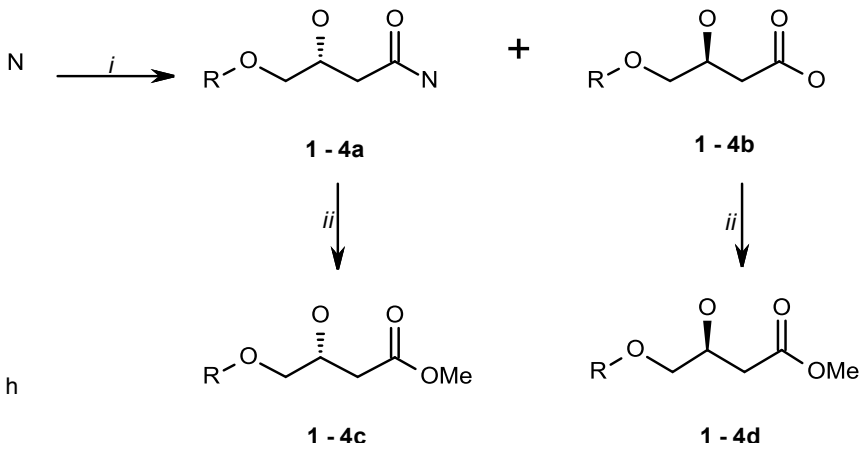


Figure 4.3: Hydrolysis of 3-hydroxy-4-phenoxybutanenitrile to corresponding amide and acid.

(i) *R. rhodochrous* sp., MeOH/ potassium phosphate buffer (1:10), 30 ºC, 1.5 h–3 h and (ii) conc. H_2SO_4, MeOH, 70°C, 1 h–overnight.

The synthesised compounds were validated using ^{13}C NMR, ^{1}H NMR as well as LCMS analysis. All reaction compounds were solubilised in low volumes of methanol (<10% v/v) before making up to the required volume using either a phosphate buffer (100mM, pH 7.0 for all β-hydroxy nitrile reactions), or a Tris-HCl buffer system (100mM, pH 9.0 for all β-amino nitrile reactions). Substrate stability testing was done at the onset to ensure compound stability during the reaction.

4.2.5 Reaction conditions

Wet cells (0.9-1.0 g) were added to a solution containing 100 mg of the respective β-amino nitrile (10ml final volume, 100mM Tris HCl buffer, pH 9) or β-hydroxy nitrile substrate (10 ml final volume, run in 100mM phosphate buffer, pH 7). The substrates were first solubilised in 1 ml methanol prior to the addition of the respective buffers and cells. The resulting mixture was then stirred at 30ºC with amide and acid generation being monitored initially by TLC. The reactions were quenched by centrifugation at

15,344 × g for 15 min with the supernatant being decanted and extracted with ethyl acetate.

4.2.6 Reaction sample processing

Reaction samples were quenched at varied time intervals (0 min - 24h) with the supernatant being extracted using three volumes of ethyl acetate. Both substrate and product recovery was attempted by washing the cellular pellet with methanol. The extracted supernatant fraction and the cellular pellet extract were then combined, and water and brine washed before drying over anhydrous magnesium sulphate. Concentration was performed under reduced pressure with low temperature to minimise thermal decomposition of the samples. Flash column purification of the amides and nitriles were done using silica gel 60 F_{254} plates. Extract purification was done by flash column purification using varied ratios of hexane (Hex)/ ethylacetate (EtOAc) for the β-hydroxy nitrile reactions or both hexane/ethyl acetate as well as dichloromethane (DCM)/ methanol (MeOH) solvent systems in the case of the β-amino nitrile reactions tested.

Extract purification of β-hydroxy nitrile reactions were started with a Hex/ EtOAc solvent system (20: 80, %v/v), then increased gradually to a final volume of 100% EtOAc. Nitrile eluted first at lower polarity, followed by amide and the acid. Purification of β-amino nitriles reactions was achieved using a 40 cm flash column, packed with silica gel (silica gel 60) and eluted with a Hex: EtOAc (50: 50, %v/v) solvent mixture, increased gradually up to 100% EtOAc to remove the β-amino nitrile. Once the nitrile was removed the solvent system was changed to MeOH/ DCM (5:95, %v/v) and increased to 20% MeOH to elute the β-amino amide. Sample analysis was by HPLC, as well as NMR and LCMS analysis (Kinfe *et al*., 2009; Chhiba *et al*., 2012).

4.2.7 Analytical methods

4.2.7.1 Standard curve data for activity quantification

All substrates and products used for activity quantification were solubilised in methanol and analysed by HPLC.

4.2.7.2 Analytical methods for β-amino nitriles and amides

Non-chiral nitrile and amide analysis was performed using HPLC system composed of Waters 2690 separation module (Waters, Milford, MA, USA) coupled with Waters Diode Array Detector 996 (210 nm to 400 nm), a Waters X-Terra MS C18 3.5 µm, 3.0 × 50 mm (i.d x L) column at 25ºC, with an isocratic eluent of 0.1% v/v TFA in ACN (composition was varied according to the compound analysed) at a flow rate of 0.3 $ml.min^{-1}$ to 0.5 $ml.min^{-1}$. The run time was 15 - 20 min. All data handling was by Empower 2 Software.

Chiral nitrile analysis was performed using a liquid chromatography system composed of a Waters 600-MS Separation Module equipped with Waters 717 Autosampler, Waters 2486 UV/Visible Detector (210 nm) and Empower 2 software. Columns used were a Chiralpack®AD-H, 250 × 4.6 mm, 5 µm and a Chiralcel® OD-H, 250 × 4.6 mm, 5 µm (Daicel Chemical Industries Ltd., Japan) at 25ºC. The eluent was Hexane (Hex): isopropanol (IPA) (both HPLC Grade, 90:10, %v/v) and an isocratic flow rate of $1ml.min^{-1}$. The run time was 30 min. The chromatographic system was conditioned for at least 1 h in advance with a column flow rate of 1 $ml.min^{-1}$ before the injection of samples.

Chiral amide analysis was performed using a liquid chromatography system composed of a Waters 2690 Separation Module equipped with Photodiode Array Detector 996 (210nm), and Empower 2 software. The column was a Crownpak CR(+), 150 × 4 mm (10ºC). The eluent used was 16.3 $g.l^{-1}$ perchloric acid, pH adjusted 2, in nano-pure water (MilliQ) at a flow rate of 0.25 $ml.min^{-1}$ over a run time of 20 to 60 min. The

chromatographic system was conditioned for at least 1 h with a column flow rate of 0.25 $ml.min^{-1}$ before the injection of samples.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded as either deuteriochloroform or deuteriomethanol solutions with tetramethylsilane as an internal standard. Both a 400 MHz Varian Unity spectrophotometer (Varian Incorporation, USA) as well as a 200 MHz spectrophotometer were used for all substrate and product analysis. The carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instruments using tetramethylsilane (TMS) as an internal standard.

β-Hydroxy nitrile reactions

To determine the enantiomeric excess (ee) values, the amides and acids produced by the biotransformation were esterified using Fischer esterification. After completion of the reaction, the reaction mixture was cooled down to room temperature and then extracted with diethyl ether. The combined organic layers were washed successively with water, 5% aqueous Na_2CO_3, and brine; dried over anhydrous $MgSO_4$; and then concentrated to dryness to afford the corresponding esters.

The ee values of esters derived from the corresponding amides and acids were determined by chiral HPLC analysis using Chiralcel® HPLC columns (Daicel Chemical Industries Ltd., Japan) OB-H, AD-H, and OD-H with a dimensions of 46 mm × 250 mm, (i.d. × L), on a Waters Alliance HPLC system (2690 Separation Module, 996 PDA detector), at a flow rate of 1 $ml.min^{-1}$ with 90:10, Hex: IPA as mobile phase unless otherwise indicated. The column temperature was maintained at 25°C using a ThermaSphere TS-430 temperature control unit (Phenomenex, USA). The detection wavelength was set at 254 nm. The absolute configuration of the products was assigned on the basis of optical rotation and elution order in chiral HPLC as compared to that reported in the literature.

4.2.8 Enantiomeric excess and % conversion data

The ee of the enzyme being studied was determined using the following equation: %eeS or %eeP = {(R-S)/ (R+S)}*100, where R and S denote the two possible enantiomer compounds from the racemate substrate (S) initially used, as well as the enriched nitrile or selective amide (P) generated in the reaction process. The % conversion of each reaction was calculated as follows: % conversion= (mmol P)/ (mmol P+ mmol S).

4.3 Results and Discussion

4.3.1 Biocatalysis monitoring

Biocatalysis reactions were monitored using the reverse phase HPLC method developed in chapter 2, sub-section 2.4.1 page 41.

4.3.2 Method development of non-chiral and chiral separation and quantification on β-substituted nitrile compounds.

Non- chiral methods

Improved reversed-phase HPLC resolution of benzonitrile and its corresponding amide and acid were separated in Waters X-Terra MS C18 3.5 µm, 3.0 × 50 mm (i.d×L) column, with run time of less than 3.5 min, has been reported in chapter 2. Good resolution of β-amino nitriles and β-amino amides studied was not achieved with this method. A modified non-chiral HPLC method to quantify all racemic β-substituted nitriles and corresponding β-substituted amides was required.

Method development was done using a mobile phase consisting of 0.1% TFA in water with ACN in various proportions at different flow rates and constant column temperature (25ºC). Mixtures of all β–substituted nitriles synthesised and their corresponding amides and acids (3-amino-3-phenylpropionic and 3-hydroxy-4-phenoxybutyric acids) were

used during method development. The optimisation chromatographic conditions obtained are summarised in table 4.1. The optimum baseline separation conditions of the analytes on Waters X-Terra MS C18 3.5 µm were achieved by varying the organic modifier, ACN, between 10% and 20% (v/v).

A change in the elution order of 3-amino-3-phenylpropionic and 3-amino-3-phenylpropionitrile, using constant chromatographic conditions (that is, flow rate, column temperature and injection volume) was observed (figure 4.4).

Table 4.1:Chromatographic conditions for the analysisof β-substituted nitriles and their related acids and amides studied.

Analytes	Absorbance Wavelength (nm)	Mobile Phase composition (%v/v)	Flow rate (ml. min^{-1})
Benzamide[a] 3-Amino-3-Phenylpropanamide 3-Amino-3-Phenylpropionic acid 3-Amino-3-Phenylpropanenitrile	210	0.1% TFA : ACN (98:2)	0.4
Benzonitrile[a] 3-Hydroxy-4-Phenoxybutanamide 3-Hydroxy-4-Phenoxybutyric acid 3-Hydroxy-4-Phenoxybutanenitrile	220	0.1% TFA : ACN (80:20)	0.3
3-Amino-3-(4-Methylphenyl) propannamide, and 3-Amino-3-(4-Methylphenyl) propanenitrile	210	0.1% TFA : ACN (90:10)	0.5
3-Amino-3-(4-Methoxyphenyl) propanamide, and 3-Amino-3-(4-Methoxyphenyl) propanenitrile	225	0.1% TFA : ACN (90:10)	0.5
3-Amino-3-(3-Bromophenyl) propanamide, and 3-Amino-3-(3-Bromophenyl) propanenitrile	225	0.1% TFA : ACN (90:10)	0.5
4-Amino-3-(4-Bromophenyl) propanamide, and 4-Amino-3-(4-Bromophenyl) propanenitrile	225	0.1% TFA : ACN (90:10)	0.5

[a] Internal standard

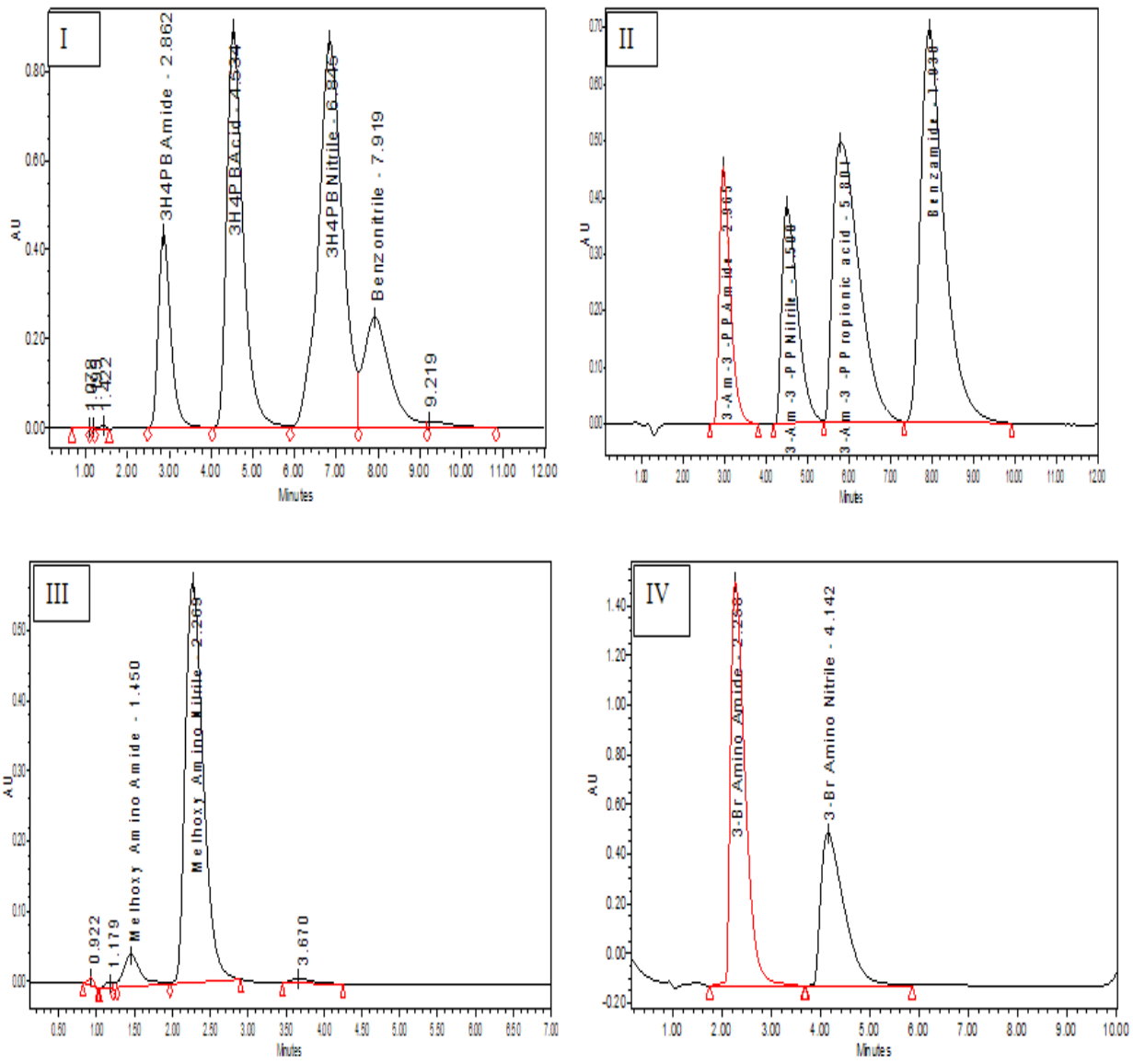


Figure 4.4:Typical chromatograms showing separation of various β-substituted nitrile mixtures components.

I - R_t = 2.862 (3-hydroxy-4-phenoxybutanamide), R_t = 4.534 (3-Hydroxy-4-phenoxybutanoic acid), R_t = 6.846 (3-Hydroxy-4-phenoxybutanenitrile) and R_t = 7.919 (benzonitrile); **II** - R_t = 2.965 (3-amino-3-propanamide), R_t = 4.534 (3-amino-3-propanenitrile), R_t = 6.846 (3-amino-3-propionic acid) and R_t = 7.919 (benzamide); **III** - R_t = 1.150 (3-amino-3-(4-methoxyphenyl) propanamide and R_t = 2.269 (3-amino-3-(4-methoxyphenyl) propanenitrile; IV – R_t = 2.268 (3-amino-3(3-bromophenyl) propanamide and R_t =4.142 (3-amino-3-(3-bromophenyl) propanenitrile). Chromatographic conditions: Waters X-Terra MS C18 3.5 µm, 3.0 × 50 mm (i.d × L) column (25ºC), with eluent of 0.1% v/v TFA in ACN (composition was varied according to the compound analysed) at an isocratic flow rate between 0.3 ml.min^{-1} and 0.5 ml.min^{-1}.

Generally in RP-HPLC, the retention of the highly water soluble amines occurs at an alkaline pH when using a hydrophobic column such as Waters X-terra MS C18. Thus the observed reversal elution order may be due to protonation of amino groups of the nitrile resulting in ammonium ions not interacting with the stationary phase efficiently, and consequently the nitrile was eluted rapidly. All developed methods were applied in quantification of biocatalytic reactions catalysed by *R. rhodocrous* ATCC BAA-870.

Chiral separation of β-amino nitriles and amides

Pure enantiomer of β-amino amides and acids can be obtained through biocatalysis of related nitriles (figure 4.5). In this study we used *R. rhodocrous* ATCC BAA-870 cells which had been shown to possess a nitrile hydratase/ amidase biocatalytic system (Brady *et al.*, 2004). Thus we attempted to separate each nitrile and its hydrolysis products within a single chromatographic run. Unfortunately this goal was not achieved, and each compound was resolved separately.

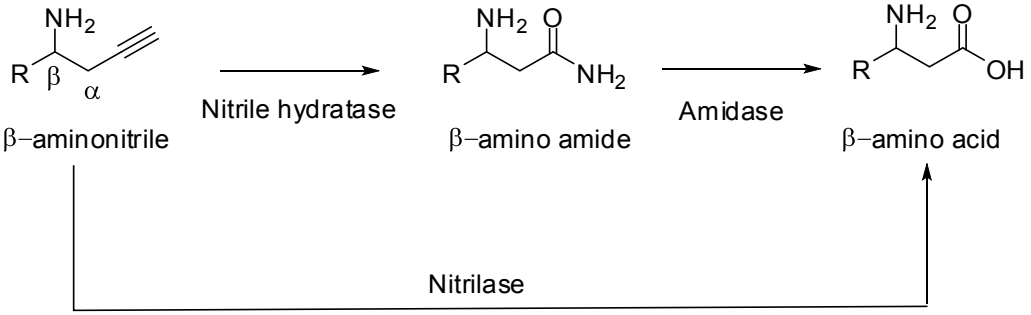


Figure 4.5: Pathways of biocatalysis of β-amino nitrile.

Polysaccharide based columns, Chiralcel® AD and Chiralpak® OD, are the most widely applied for chiral separation of β-substituted nitrile compounds (Winkler and Klempier, 2009). For this reason Chiralpak® AD-H and Chiralcel OD-H, which have enhanced chiral selectors as AD and OD (Chiral Technologies, 2003), respectively, were selected for quantification of all enantiomers of racemic β-amino nitrile and amides studied. The appropriate mobile phase for each of analytes was selected by first running the sample using the weak mobile phase concentrations (table 4.2). Thereafter, the solvents

compositions were adjusted to weaker or stronger mobile phase compositions, accordingly, guided by retention times, for reasonable enantioseparation analysis times.

Table 4.2: Solvent mixtures for new method development.

	n-Hexane: Isopropanol	n-Hexane: Methanol	Hexane: Ethanol
Weaker	95:5	98:2	95:5
Moderate	90:10	95:5	90:10
Stronger	80:20	90:10	

Figure 4.6 shows selected chromatograms of enantiomeric separation of racemic β-amino nitrile on a Chiralpak® AD-H and Chiralcel® OD-H columns at 210 nm wavelength detection. Neither MeOH nor EtOH as an alcohol in the mobile phase could yield separation. This could be due to the higher polarity of the two alcohols as compared to IPA.

Crownpak CR(+) column contains chiral crown ether as a chiral selector and can separate compounds bearing a primary amino group near the chiral centre (Winkler and Klempier, 2009; Daicel Crownpak CR(+) Instruction Manual, 1995). The separation was carried out using perchloric acid (16.3 $g.l^{-1}$) in water with pH adjusted 2, as mobile phase. The variation of the mobile phase pH is one of the factors that govern the separation. The lower pH improves the conditions for complex formation, but at the same time shortens column life span [Daicel Crownpak CR(+) Instruction Manual, 1995], thus a mobile phase at pH 2 was selected as optimal pH.

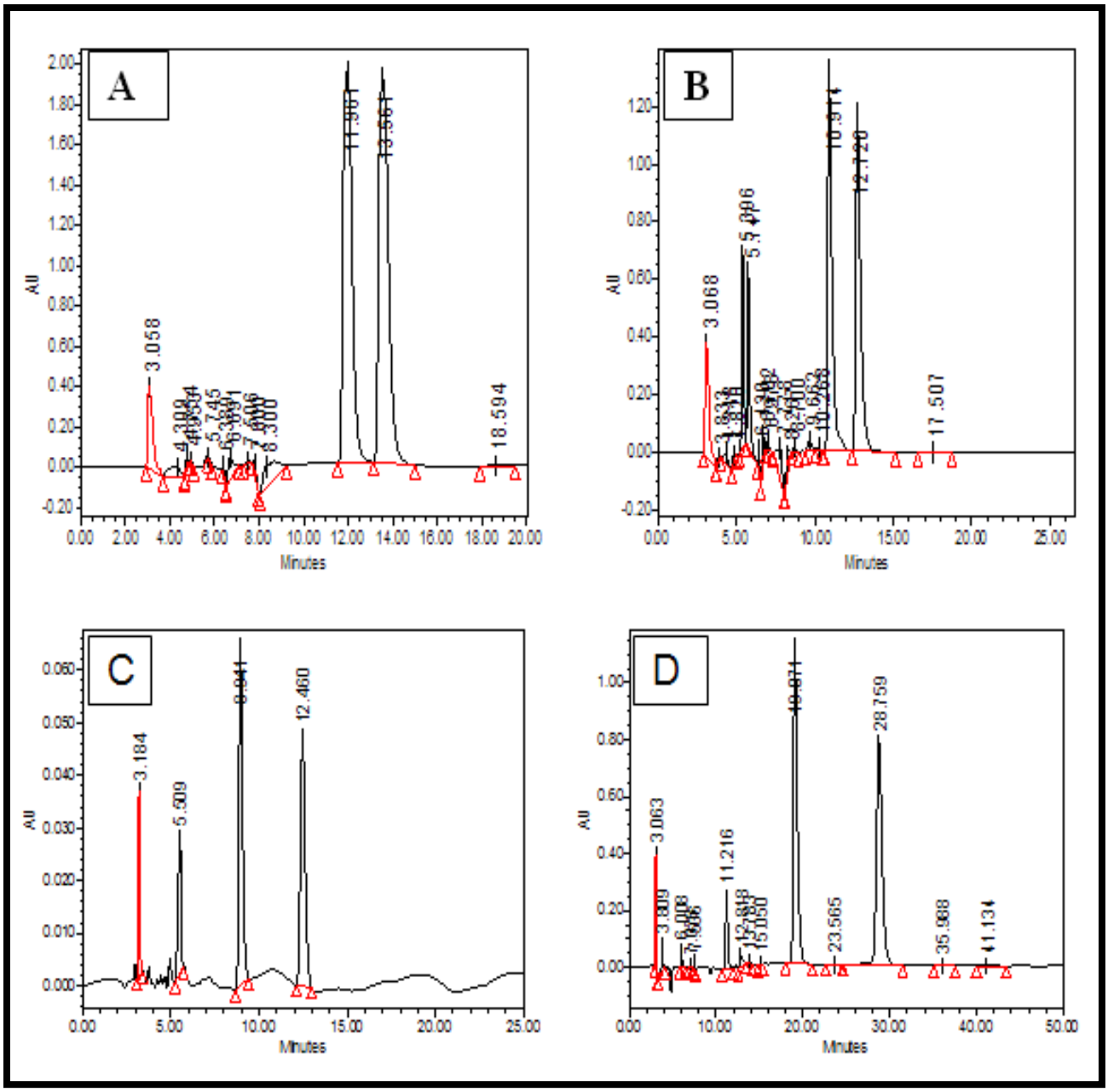


Figure 4.6:Typical chromatograms showing enantiomeric separation of various racemic β-amino nitrile substrates.

(A), 3-amino-3-phenyl propanenitrile, 11.96 min (S) and 13.56 min (R); (B), 3-amino-3-(4-methylphenyl) propanenitrile, 10.91 min (S) and 12.72 min (R); (C), 3-amino-3-(4-chlorophenyl) propanenitrile, 8.94 min (S) and 12.46 min (R); (D), 3-amino-3-(3-bromophenyl) propanenitrile, 19.07 min (S) and 28.76 min (R) results. Chromatographic conditions: Chiralpack® AD-H, 250 × 4.6 mm, 5 µm (A, B and C);Chiralcel® OD-H, 250 × 4.6 mm,5 µm (D); mobile phase:- Hex: IPA (90:10, v/v); flow rate:- 1 ml.min^{-1}; detection wavelength:- 210 nm; injection volume:- 10 µl; column temperature:- 25ºC; run time 30 min.

With perchloric acid in water at pH 2 as mobile phase, ambient temperature and flow rate of 1 $ml.min^{-1}$, only 3-amino-3-phenyl propanamide and 3-amino-3-(4-methoxyphenyl) propanamide enantiomers were separated partially; thus the optimisation of the analyses was necessary. In a series of the experiments, flow rate was decreased from 1 $ml.min^{-1}$ to 0.25 $ml.min^{-1}$. This change improved the resolution. Decreasing the temperature from 25ºC to 10ºC, and keeping flow rate constant at 0.25 $ml.min^{-1}$, led to optimal resolution for all β-amino amides (table 4.3).

Table 4.3: Chromatographic conditions for separating of alicyclic β-amino amides on Crownpak CR(+).

Compound	Reagent	Column	Eluent	Flow rate and run time	Detection wave length	Temp.
Alicyclic β-amino amides	Perchloric acid nanopure water	Crownpak CR(+)	Perchloric acid, pH 2.0 in water	0.25 $ml.min^{-1}$ 20 - 40 min	210 or 220 nm	10ºC

The Crownpak CR (+) column was able to successfully resolve β-amino amide compounds (3-amino-3-phenylpropanamide and derivatives) (figure 4.7).

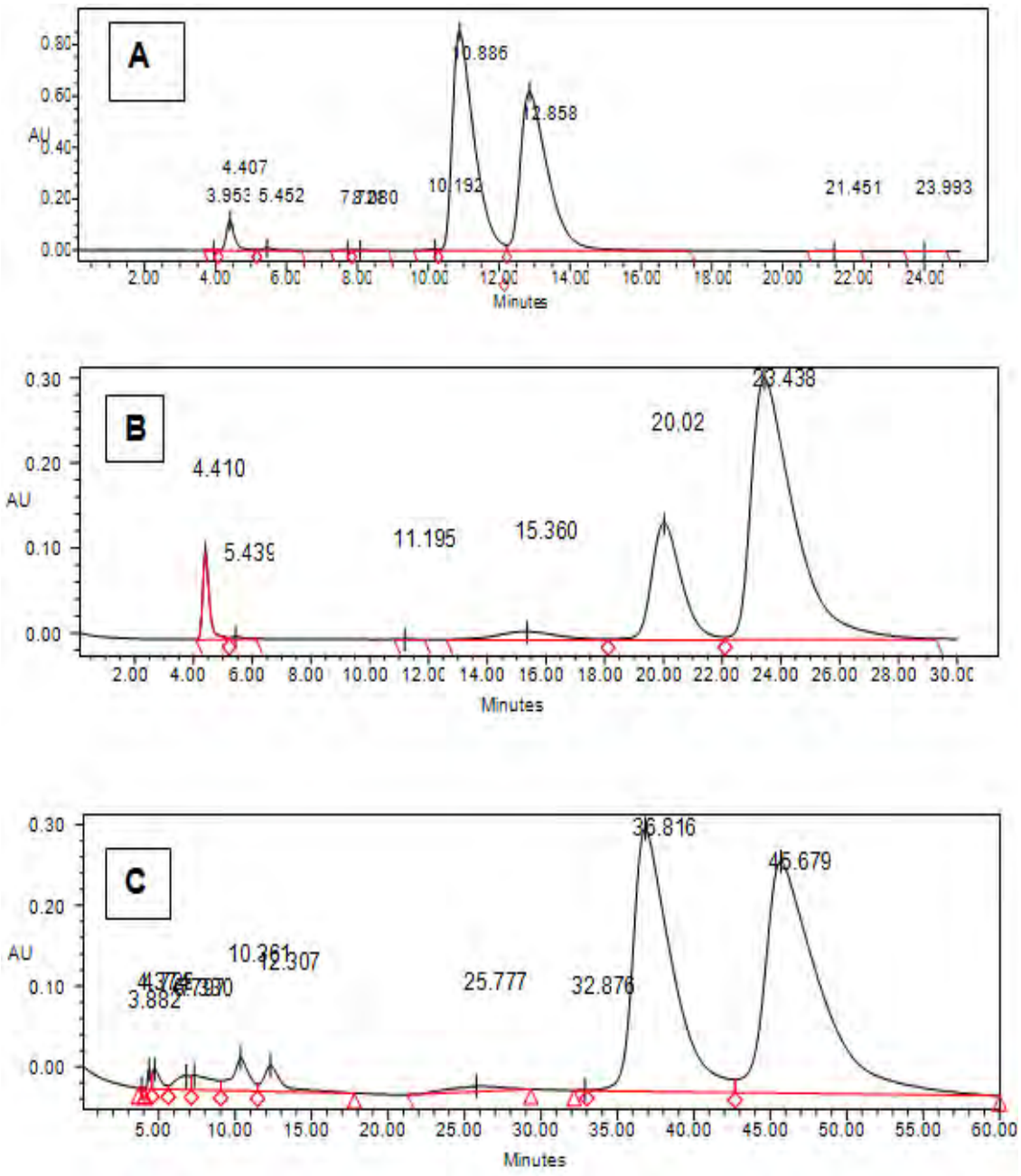


Figure 4.7: Typical chromatograms showing enantiomeric separation of β-amino amide reaction

(A), 3-amino-3-phenyl propanamide, 10.89 min (S-enantiomer) and 12.86 min (R-enantiomer); (B), 3-amino-3 (4-methoxyphenyl) propanamide, 20.02 (S-enantiomer) and 23.44 min) (R-enantiomer); (C), 3-acetylamino-3(4-chlorophenyl) propanamide, 36.82 min(S-enantiomer) and 45.68 min (R-enantiomer). Chromatographic conditions: column Crownpak CR (+); mobile phase, $HClO_4$ in water, pH 2.0; flow rate, 0.25 ml.min^{-1}, detection wavelength, UV 210 nm (A and B) and UV 220 nm (C).

Assigning of the two enantiomer peaks was done by comparing the elution times of the N-Boc‟d methoxy amino amide that was submitted for polarimetry and the spin data was compared to literature (Gonzalez *et al*.,2010), The major peak was found to be the S-amide peak.

Chiral separation of β-hydroxy nitriles and their hydrolytic products

In this study, polysaccharide based columns Chiralpak® AD-H, Chiralcel OD-H and Chiralcel OB-H (cellulose backbone phase) were evaluated for separation and quantification of all enantiomers of β-hydroxy amides, acids and their corresponding esters (where necessary). Separation of each nitrile and its hydrolysis products within a single chromatographic run was attempted, starting with of 3-hydroxy-4-phenoxybutanamide (3H4PBN) and its hydrolysis products using standards.

Figure 4.8 show resolution of 3H4PBN enantiomers and that of its hydrolysis products on the Chiralcel® AD-H column, achieved by programmed gradient flow as follows: initial flow rate was 0.5 $ml.min^{-1}$, increased to 1 $ml.min^{-1}$ over 30 min held for 5 min. The mobile phase ratio, Hex: IPA (95:5, %v/v) remained the same throughout the run, and the detection was done at wavelength of UV 254 nm. The second enantiomer of 3-hydroxy-4- phenoxybutanamide (3H4PBAm) was found to be relatively broad. This may be attributed to its polarity which enhances its interaction with the stationary phase charged groups, delaying its elution.

The most interesting observation from the chiral HPLC method developed for the separation of 3H4PBN enantiomers and that of its hydrolysis products on a single chromatographic run, is that variation of flow rate, just like alteration of the type and concentration of the organic modifier, could also have a beneficial effect on the resolution of enantiomers on polysaccharides based CSPs. Árki *et al*. (2006) reported the same observation in their study on direct separation of underivatised β-amino acids on a macrocyclic glycopeptide antibiotic teicoplanin (Chirobiotic T) column and ticoplanin aglycone (Chirobiotic TAG) column.

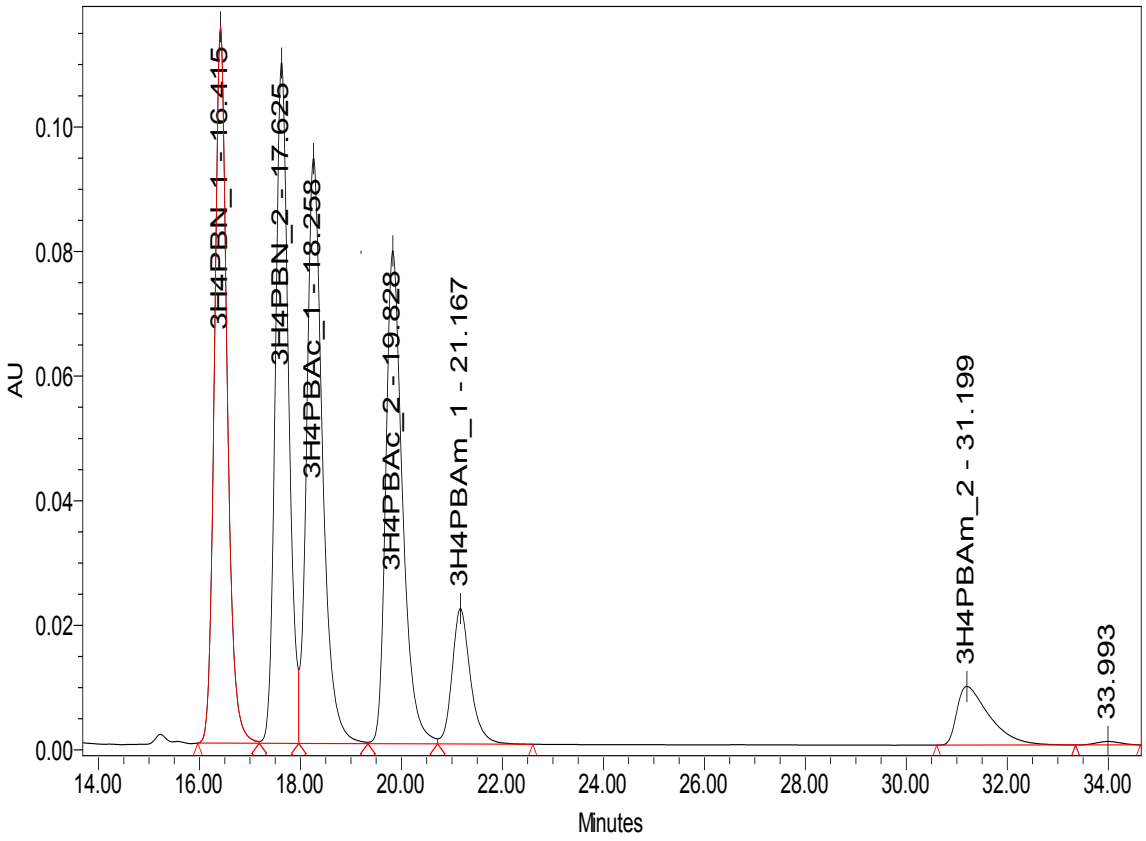


Figure 4.8: Chromatogram showing the chiral separation of 3H4PBN, 3-hydroxy-4-phenoxybutanoic acid (3H4PBAc) and (3H4PBAm) enantiomers on Chiralcel® AD-H column

Mobile phase, Hex: IPA (95:5, %v/v); gradient flow: starting at 0.5 ml.min^{-1} and gradually ramping it to 1 ml.min^{-1} in 30 min, then hold at 1 ml.min^{-1} for 5.min; thermostated column temperature, 25ºC; detection wavelength UV 254 nm.

The optimum chromatographic conditions for enantioseparation of β-hydroxy nitrile compounds and their hydrolytic products analysed separately, were obtained using the same strategy used for β-amino nitriles and amides. The detection wavelength was performed at UV 254 nm. All experiments were performed with a pump in isocratic mode.

Most of the β-hydroxy amides, acids and esters studied were separated in Chiralcel OD-H® (table 4.4), except (R)-(+)-3-hydroxy-4(4-methoxyphenoxy)butanoate, methyl-(R)-(+)-3-hydroxy-3-(4-methylphenyl)propanoate, and methyl-(R)-(-)-3-hydroxy-3-(4-methylphenyl)propanoate, which were successful separated in Chiralpak AD-H® and Chiralcel OB-H®, respectively.

Table 4.4: Retention times of β-hydroxy esters enantiomers prepared from an acid or amide and separated on Chiralpak® AD-H, Chiralcel® OB-H or OD-H.

	Enantiomer retention time, R_t (min)				
	Acid		Amide		
Analyte	R	S	R	S	Column
Methyl-3-hydroxy- 3-phenylpropanoate	10.75	12.66	10.74	12.65	OD-H
Methyl-3-hydroxy- 3-(4-methylphenyl)propanoate	15.95	19.05	15.03	18.75	OB-H
Methyl-3-hydroxy-4-(4-methoxyphenoxy)butanoate	17.72	19.75	16.74	18.69	AD-H
Methyl-3-hydroxy-4-phenoxybutanoate	12.84	14.57	13.02	14.82	OD-H
Methyl-3-hydroxy-4-benzyloxybutanoate	12.44	15.21			OD-H
[a]Methyl-3-hydroxy-4-(4-chlorophenoxy)butanoate	12.97	11.01	12.93	11.15	OD-H

Mobile phase, Hex: IPA (90:10, %v/v); flow rate 1.00 ml.min^{-1}, thermostated column temperature, 25ºC; Wavelength detection, UV 254nm.

[a] Elution order of enantiomers is reversed

4.3.3 Analytical data for quantification of reaction products

Standard curves of the nitrile hydratase catalysed reaction substrates and products studied were constructed: 3-amino-3-phenyl propanamide, the product of 3-amino-3-phenyl propanenitrile; 3-amino-3-(4-methylphenyl) propanamide, the product of 3-amino-3-(4-methylphenyl) propanenitrile; 3-hydroxy-4-phenoxybutanamide, the product of 3-hydroxy-4-phenoxy butanenitrile (1-5 mM). Refer to figure 4.9.

All concentrations were made up in 100% methanol and sealed in HPLC vials immediately prior to analysis to avoid sample concentration due to solvent volatilization. Graphs were generated using duplicate sample points per data point.

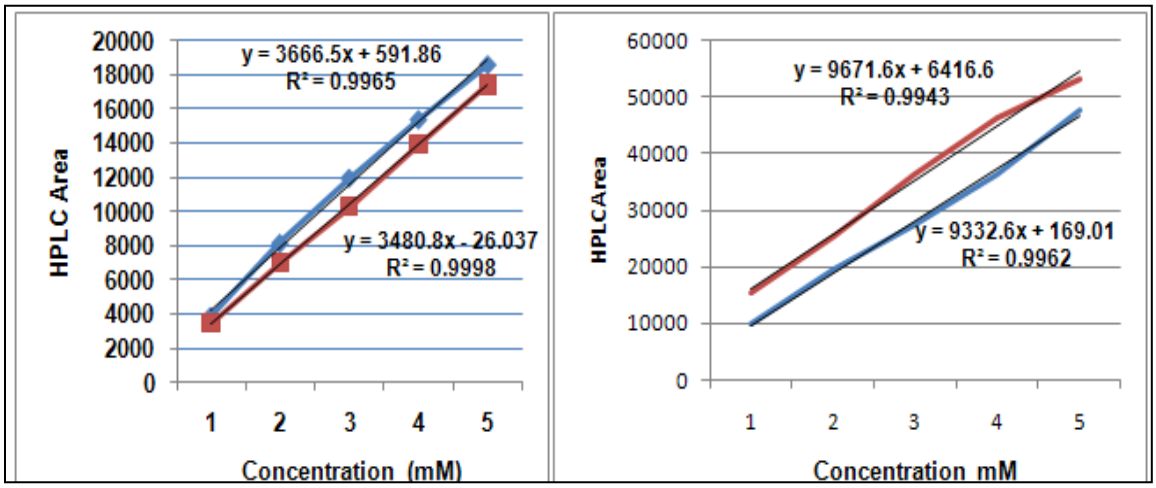
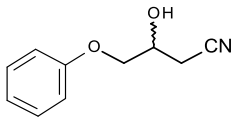
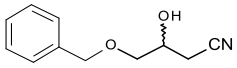
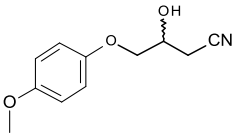
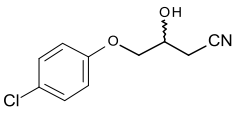


Figure 4.9: Selected HPLC standard curves for (♦) 3-amino-3-phenylpropanenitrile and (■) 3-amino-3-phenylpropanamide; (—) 3-amino-3-(4-methoxyphenyl) propanenitrile and (—) 3-amino-3-(4-methoxyphenyl) propanamide (1-5 mM).

The percentage conversions (table 4.5 and 4.6) of the β-substituted nitrile whole cell reactions were determined by HPLC using the constructed β-substituted nitrile, β-substituted amide, and β-hydroxy acids standard curves. All reactions were run at 30ºC.

Table 4.5 results show that the biocatalyst *R. rhodochrous* ATCC BAA-870 hydrolysed β-hydroxy nitrile enantioselectively when the organism was incubated at 30°C in 100 mM phosphate buffer of pH 7.2, providing enantiomeric excess ranging from 82% to 99% of the (S)-acid, although in a case of 4-benzyloxy-3-hydroxy-butanenitrile the acid produced enantioselectively was (R)-acid. The highest enantioselectivity was found in the conversions of 4-(4-methoxyphenoxy)-3-hydroxybutanenitrile and 4-(benzyloxy)-3-hydroxybutanenitrile to their respective amides. This high enantioselective biocatalysis of *β*-hydroxy nitriles is in accordance with literature reports (Wu and Li, 2003; Kubáč *et al*., 2008).

Table 4.5: Synthesis of acyclic β-hydroxy acids, showing substrate conversion and enantiomeric excess of acids.

Compound name	**β-subsituted nitrile substrate**	**Conversion (%)**	**Acid enantiomeric excess (%)**
3-hydroxy-4-phenoxy-butanenitrile		32	99 (S-Acid)
4-benzyloxy-3-hydroxy-butanenitrile		14	99 (R-Acid)
3-hydroxy-4-(4-methoxyphenoxy) butanenitrile		14	96 (S-Acid)
4-(4-chlorophenoxy)-3-hydroxy-butanenitrile		24	82 (S-Acid)

The high enantiomeric excess values of the acids indicate that amidase involved in *R. rhodochrous* ATCC BAA-870 is enantioselective toward β-amino nitriles. Enantiomer preference observed could be attributed to presence of metal cation on the active side of the enzyme. Enantioselectivity studies by Van Pelt (2010) suggest that cobalt containing Nhase from *Rhodopseudomonas palustris* HaA2, *Rhodopseudomonas palustris* CGA009, *Sinorhizobium meliloti* 1021 and *Nitriliruptor alkaliphilus* are enantioselective for α-amino acids, while Fe-containing nitrile hydratase from *Rhodococcus erythropolis* AJ270 is not enantioselective.

Functionalising 3-hydroxy-4-phenoxy-butanenitrile by introducing electrons accepting group, methoxy-, on the aromatic ring produced enantiomeric ee of 96% of the (S)-acid. Studies on substrate preference of amidase suggest that *Rhodococcus* strain was more stereoselective for large molecules (Wang and Feng, 2002; Wang and Feng, 2003). Addition of electron withdrawing substituents on the aromatic ring resulted in 82% ee. This is in agreement with the results reported by Wu and Li (2003).

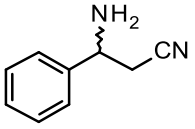
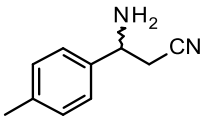
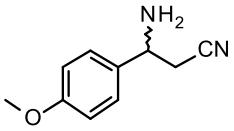
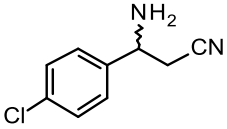
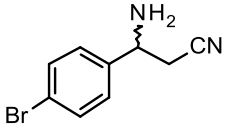
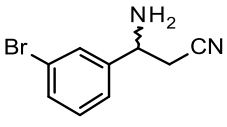
Initially, enantioselective hydrolysis of unprotected β-amino nitriles was attempted at neutral pH. Unfortunately, no conversion of nitrile to either amide or acid was observed even after 72 h incubation. However, when protecting the amino group with N- acetyl, N- tosyl or N-BOC, biocatalysation of β-amino nitriles to corresponding β-amino amides with N- acetyl was observed. The optimisation of buffer and pH buffer, Tris-HCl and 9.0, respectively, resulted in hydrolysis of unprotected nitriles (Chhiba *et al.*, 2012). The ee of the purified nitriles and amides were determined by chiral HPLC after confirmation of structure with ^{1}H and ^{13}C NMR and LCMS.

Table 4.6 show 46% conversion of the 3-amino-3-phenyl propanenitrile (1 h reaction) with an amide ee of 7% (S). Reactions carried out using 3-amino-3-(4-methylphenyl) propanenitrile showed a 39% conversion with a 3% ee (S) on the amide. A 62% conversion of 3-amino-3-(4-methoxyphenyl) propanenitrile after an hour with the amide of 24% ee (S) was achieved. Addition of electron withdrawing substituents on the aromatic ring generated better enantioselectivity as compared to the parent compound,

3-amino-3-phenyl propanenitrile, excluding 3-amino-3-(3-chlorophenyl) propanamide. Although the 3-Cl substituted nitrile and amide were not determined, the biocatalysis of 3- and 4-Br substituted nitriles to corresponding amides is in agreement with our previous studies with β-hydroxynitriles (Kinfe *et al*., 2009).

The biocatalytic synthesis of acyclic β-amino amides show similar trends observed with synthesis of acyclic β-hydroxy acids, except that the enantiomeric excess results were very low as compared to that of the β-hydroxy acid. This low enantioselectivity of the biocatalysis, indicate that the nitrile hydratase expressed by *R. rhodochrous* ATCC BAA-870 displays lower enantioselectivity toward β-amino nitriles. Further, it was observed that nitrile hydratase was enantioselective towards β-amino nitriles, which was not apparent in the biocatalysis of β-hydroxynitriles.

Table 4.6: Synthesis of acyclic β-amino amides, showing substrate conversion and enantiomeric excess.

Compound name	Reaction time (min)	β-subsituted nitrile substrate	Conversion (%)	Amide enantiomeric excess (%)
3-amino-3-phenyl propanenitrile	60		46	7 (S-Amide)
3-amino-3-(4-methylphenyl) propanenitrile	60		39	3 (S-Amide)
3-amino-3-(4-methoxyphenyl) propanenitrile	60		62	24(S-Amide)
3-amino-3-(4-chlorophenyl) propanenitrile	72		0	ND(Amide)
3-amino-3-(4-bromophenyl) propanenitrile	60		55	43(S-Amide)
3-amino-3-(3-bromophenyl) propanenitrile	60		33	22 (S-Amide)

4.4 Conclusion

A general approach for non-chiral separation of β–substituted nitriles and β–substituted amides was developed on a Waters X-Terra reversed phase column by means of adjusting the eluent according to the specific compounds under analysis.

The biocatalytic asymmetric hydrolysis of β-substituted nitriles to corresponding β-substituted amides and acids was studied. Chiral separation of both enantiomers present in the β–amino- and β–hydroxy-compounds used in this study was achieved on chiral stationary phases Chiralpak® AD-H, Chiralcel® OB-H and Chiralcel® OD-H. β-amino amide enantiomers were determined using a Crownpak CR(+) column.

Using these techniques it was possible to determine the conversion and the enantiomeric excess achieved by biocatalytic hydrolysis of nitriles by *R. rhodochrous* ATCC BAA-870. Enantiomeric excesses of more than 99% was observed with the nitrile metabolism of aromatic β–hydroxy nitriles, while enantioselective hydrolysis β–amino nitriles, albeit at lower ee values up to 42%, was observed for the first time. The organism, *R. rhodochrous* ATCC BAA-870, expressed nitrile hydratase and amidase activities responsible for these biotransformations.

Chapter 5: Conclusions

5.1 Introduction

Nitriles are used in industrial organic synthesis as important chemical precursor to amides and acids, for example in the industrial production of bulk polymers as well as enantio-pure pharmaceutical compounds for anti-cancer, antiviral, ACE inhibitors and many more drugs. Nitrile can be prepared synthetically via a variety of routes.

Non-catalytic organic synthetic routes have associated problems, such as unwanted by-products due to in some cases due to low chemo-, stereo- or regio-selectivity. This results in low yields, and high consumption of energy, thus they are neither eco-friendly or optimally economical. Nitrile biocatalysis is an attractive and commercially proven alternative. Biocatalysis of nitrile follows two routes to produce organic acids. The first route, is a single step process catalysed by nitrilase (EC 3.5.5.1) (Harper, 1977), and the second pathway involve nitrile hydratase (EC 4.2.1.84) and amidase (EC 3.5.1.4) catalysed sequential reaction (Asano *et al*., 1980). These three enzymes are the key catalysts in nitrile biocatalysis.

Nitrile hydratases are an important class of hydrolyases that converts naturally occurring, as well as xenobiotically derived, nitriles to corresponding amides. Because of their inherent enantio- and regioselectivity and other benefits, nitrile hydratases are attractive as „green", mild, and selective catalysts for setting stereogenic centers in fine-chemical synthesis and enantiospecific synthesis of amide and carboxylic acid derivatives. For example, Wu and Li (2003) demonstrated that the asymmetric hydrolysis of aromatic β-hydroxy nitriles could be achieved with moderate to high yield, using *Rhodochrous sp.* CGMCC 0497. Kamal and Khanna (2001), have also demonstrated biocatalytic resolution of 4-aryloxy-3-hydroxybutanenitriles with excellent enantiomeric excess of up to 99%, however that synthetic route involved the strategy of hydrolysing the corresponding esters using lipase from *Pseudomonas cepacia*.

To determine the stereoselectivity of the of nitrile hydrolysis, enantio-separation of the enzyme substrates and products by HPLC is a key analytical technique. This study was initiated to developed non-chiral and chiral HPLC methods for quantification of compounds present in nitrile biocatalysis reactions, with the biocatalyst prepared in various forms; that is whole cells of *Rhodococcus rhodochrous* BAA-870, partially purified nitrile hydratase, and self-immobilised nitrile hydratase(using the SphereZyme™ technique); and to evaluate the application of whole cell biocatalyst against β-substituted chiral compounds.

5.2 A rapid reversed phase method for the quantitative determination of benzamide, benzoic acid and benzonitrile X-Terra MS (or RP) 18 3.5 µm

Non-chiral methods had been developed and optimised for the separation of reaction compounds, benzamide, benzoic acid and benzonitrile. The resolution of these compounds was achieved using X-Terra MS C18 3.5 µm column. The improved development of the RP-HPLC method was validated for accuracy, precision, linearity, and limit of detection and quantification. The developed method was used for the quantification of biocatalyst activity in the partially purified nitrile hydratase. This method was useful for monitoring nitrile hydratase activity during purification process, which was found to be 351.8 $U.mg^{-1}$, similar to that obtained by Van Pelt (2010).

5.3 Immobilisation of nitrile hydratase

Although only low specific activity (1.20 $U.ml^{-1}$) of the immobilised nitrile hydratase of *Rhodococcus rhodochrous ATCC BAA-870* was obtained, this was the first time that the enzyme from this species had been immobilised. It is also the first attempt at immobilisation of a nitrile hydratase using the novel SphereZyme™ self-immobilisation

technique. Continuous improvement of this enzyme immobilisation with other techniques will contribute to more applications of it as green nitrile biocatalyst.

In addition, the retention of the activity of immobilised nitrile hydratase from *R. rhodochrous ATCC BAA-870* in the presence of non-polar organics, as demonstrated in this study, provides evidence for its potential usefulness in the chemical and pharmaceutical industries for biocatalysis of nitriles to carboxyamides and carboxylic acids.

5.4 Quantification of β-amino nitrile and β-hydroxy nitrile compounds

Synthesis of various β-substituted nitriles had been carried out and the enantioselective hydrolysis of these β-substituted nitriles to the corresponding substituted products using *Rhodococcus rhodochrous* ATCC BAA-870 was demonstrated.

Chiralpak® AD-H, Chiralcel® OB-H and Chiralcel® OD-H column revealed a good enantio-separation capability for all β-substituted compounds, these CSPs complement each other. A systematic approach of the separation of β-substituted nitriles and their hydrolytic products on the three polysaccharide based CSPs was attained by varying the composition of the mobile phase. Crownpak CR(+) column proved efficient in the separation of the enantiomers of the β-amino amides only. These chiral analytical techniques allowed for the observation of the unprecedented high enantioselectivity of the amidase for carboxyamides generated by the nitrile hydratase, providing enantiopure β-hydroxy acids. It also allowed for enantiomeric excess quantification of the first enantioselective hydrolysis of β-amino amides by nitrile hydratase.

5.5 Future Prospects

We intend to improve the activity of purified nitrile hydratase from *Rhodococcus rhodochrous* ATCC BAA-870. This may involve genetic engineering to enhance the

enantio- and regioselectivity of the enzyme. It may also require improving the immobilised product (for biocatalyst recovery and recycling), including development of nitrile hydratase SphereZymes™.

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7 Appendices

7.1 Chromatographic Conditions

Table 7.1: Chromotographic conditions

Column	Mobile Phase	Type of elution	Flow Rate ($ml.min^{-1}$)	Injection volume (µl)	Temperature (ºC)	Detection wavelength (nm)	Retention Times (min)	Maximum Absorbance Wavelength (nm)	Results
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Methanol (90:10)	Isocratic	0.5	10	25	210 - 400	Benzamide, Rt = 2.387; Benzoic Acid, R_t = 7.084; Benzonitrile, R_t = 9.242	Benzamide, 226.4; Benzoic Acid, 224.1; Benzonitrile, 229.9	Benzoic acid and Benzonitrile Peaks were not baseline resolved, run time reasonable
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Methanol (90:10)	Isocratic	0.6	10	25	210 - 400	Benzamide, R_t = 1.987; Benzoic Acid, R_t = 5.801; Benzonitrile, R_t = 7.616	Benzamide, 226.4; Benzoic Acid, 224.1; Benzonitrile, 228.8	Benzoic acid and Benzonitrile Peaks were not baseline resolved, run time reasonable
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Methanol (90:10)	Isocratic	0.7	10	25	210 - 400	Benzamide, R_t = 1.702; Benzoic Acid, R_t = 4.932; Benzonitrile, R_t = 6.474	Benzamide, 225.2; Benzoic Acid, 224.1; Benzonitrile, 228.8	Benzoic acid and Benzonitrile Peaks were not baseline resolved, run time

									reasonable
X-Terra RP_{18} 3.5µm	0.1%Trifluoroacetic acid + Methanol (80:20)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 1.951; Benzoic Acid, R_t =5.728; Benzonitrile, R_t =7.293	Benzamide, 226.4; Benzoic Acid, 224.1; Benzonitrile, 228.8	Benzoic acid and Benzonitrile Peaks separation is not on the baseline, run time reasonable (+/- 9 min)
X-Terra RP_{18} 3.5µm	0.1% Formic Acid + Acetonitrile (95:5)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 2.389; Benzoic Acid, R_t = 7.036; Benzonitrile, R_t = 9.042	Benzamide, 226.4; Benzoic Acid, 228.8; Benzonitrile, 228.8	Benzoic acid and Benzonitrile Peaks were not baseline resolved, run time reasonable (+/- 9 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (90:10)	Isocratic	0.4	10	25	210 - 400	Benzamide, R_t = 2.553; Benzoic Acid, R_t = 7.685; Benzonitrile, R_t = 8.652	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Benzoic acid and Benzonitrile Peaks were not baseline resolved, run time reasonable

X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	23	210 - 400	Benzamide, R_t = 2.017; Benzoic Acid, R_t = 4.835; Benzonitrile, R_t = 6.470	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	25	210 - 400	Benzamide, R_t = 1.987; Benzoic Acid, R_t = 4.606; Benzonitrile, R_t =6.274	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	26	210 - 400	Benzamide, Rt = 1.987; Benzoic Acid, R_t = 4.606; Benzonitrile, R_t =6.175	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	27	210 - 400	Benzamide, R_t = 1.977; Benzoic Acid, R_t = 4.559; Benzonitrile, R_t =6.112	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	28	210 - 400	Benzamide, R_t = 1.973; Benzoic Acid, R_t = 4.494; Benzonitrile, R_t =6.029	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)

X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.4	10	30	210 - 400	Benzamide, R_t = 1.954; Benzoic Acid, R_t = 4.355; Benzonitrile, R_t = 5.852	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 1.993; Benzoic Acid, R_t = 4.665; Benzonitrile, R_t = 6.274	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 8 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (80:20)	Isocratic	0.6	10	25	210 - 400	Benzamide, R_t = 1.357; Benzoic Acid, R_t = 3.117; Benzonitrile, R_t = 4.164	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 6 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid +Acetonitrile (75:25)	Isocratic	0.4	10	25	210 - 400	Benzamide, R_t = 1.795; Benzoic Acid, R_t = 3.522; Benzonitrile, R_t = 4.999	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (75:25)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 1.456; Benzoic Acid, R_t = 2.842; Benzonitrile, R_t = 4.012	Benzamide, 225.2; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 5.5 min)

X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (75:25)	Isocratic	0.6	10	25	210 - 400	Benzamide, R_t = 1.228; Benzoic Acid, R_t = 2.382; Benzonitrile, R_t = 3.344	Benzamide, 226.4; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 5.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.4	10	25	210 - 400	Benzamide, R_t = 1.791; Benzoic Acid, R_t = 3.507; Benzonitrile, R_t = 5.008	Benzamide, 225.2; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 6.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 1.444; Benzoic Acid, R_t = 2.801; Benzonitrile, R_t = 3.960	Benzamide, 227.6; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 5.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.6	10	25	210 - 400	Benzamide, R_t = 1.227; Benzoic Acid, R_t = 2.801; Benzonitrile, R_t = 3.961	Benzamide, 227.6; Benzoic Acid, 229.9; Benzonitrile, 224.2	Peaks well separated, run time reasonable (+/- 4.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.4	10	26	210 - 400	Benzamide, R_t = 1.786; Benzoic Acid, R_t = 3.468; Benzonitrile, R_t = 4.918	Benzamide, 226.4; Benzoic Acid, 228.8; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 6.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.5	10	26	210 - 400	Benzamide, R_t = 1.444; Benzoic Acid, R_t = 2.776;	Benzamide, 226.4; Benzoic Acid, 229.9;	Peaks well separated, run time reasonable

							Benzonitrile, R_t = 3.916	Benzonitrile, 224.1	(+/- 5.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.6	10	26	210 - 400	Benzamide, R_t = 1.221; Benzoic Acid, R_t = 2.339; Benzonitrile, R_t = 3.284	Benzamide, 225.2; Benzoic Acid, 229.9; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 4.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.4	10	27	210 - 400	Benzamide, R_t = 1.782; Benzoic Acid, R_t = 3.427; Benzonitrile, R_t = 4.849	Benzamide, 227.6; Benzoic Acid, 228.8; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 6.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.5	10	27	210 - 400	Benzamide, R_t = 1.444; Benzoic Acid, R_t = 2.775; Benzonitrile, R_t = 3.879	Benzamide, 225.2; Benzoic Acid, 228.9; Benzonitrile, 224.1	Peaks well separated, Benzamide elute in the void volume, run time reasonable (+/- 5.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (70:30)	Isocratic	0.6	10	27	210 - 400	Benzamide, R_t = 1.213; Benzoic Acid, R_t = 2.295; Benzonitrile, R_t = 3.216	Benzamide, 226.4; Benzoic Acid, 228.8; Benzonitrile, 224.1	Peaks well separated, run time reasonable (+/- 4.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (60:40)	Isocratic	0.4	10	25	210 - 400	Benzamide, R_t = 1.496; Benzoic Acid, R_t = 2.030; Benzonitrile,	Benzamide, 226.4; Benzoic Acid, 228.8; Benzonitrile,	Peaks well separated, run time reasonable (+/- 3.0 min)

							R_t = 2.691	222.9	
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (60:40)	Isocratic	0.5	10	25	210 - 400	Benzamide, R_t = 1.214; Benzoic Acid, R_t = 1.638; Benzonitrile, R_t = 2.162	Benzamide, 225.2; Benzoic Acid, 228.8; Benzonitrile, 222.9	Peaks well separated, run time reasonable (+/- 3.0 min)
X-Terra RP_{18} 3.5µm	0.1% Trifluoroacetic acid + Acetonitrile (60:40)	Isocratic	0.6	10	25	210 - 400	Benzamide, R_t = 1.032; Benzoic Acid, R_t = 1.339; Benzonitrile, R_t = 1.750	Benzamide, 224.1; Benzoic Acid, 229.9; Benzonitrile, 222.9	Peaks well separated, run time reasonable (+/- 3.0 min), peaks slightly are fronting
X-Terra RP_{18} 3.5µm	0.02% Trifluoroacetic acid + Acetonitrile (90:10)	Isocratic	0.5	10	25	211 - 400	Benzamide, R_t = 2.118; Benzoic Acid, R_t = 3.790; Benzonitrile, R_t = 5.162	Benzamide, 224.1; Benzoic Acid, 229.9; Benzonitrile, 222.10	Peaks well separated, run time reasonable (+/- 3.0 min), peaks are slightly fronting
X-Terra RP_{18} 3.5µm	0.05% Trifluoroacetic acid + Acetonitrile (90:10)	Isocratic	0.5	10	25	212 - 400	Benzamide, R_t = 2.118; Benzoic Acid, R_t = 3.790; Benzonitrile, R_t = 5.162	Benzamide, 224.1; Benzoic Acid, 229.9; Benzonitrile, 222.11	Peaks well separated, run time reasonable (+/- 3.0 min), peaks are fronting

X-Terra RP_{18} 3.5µm	20mM KH_2PO_4 +Acetonitrile (90:10)	Isocratic	0.5	10	25	213 - 400	Benzamide, R_t = 2.118; Benzoic Acid, R_t = 3.790; Benzonitrile, R_t = 5.163	Benzamide, 224.1; Benzoic Acid, 229.9; Benzonitrile, 222.12	Benzoic Acid and Benzonitrile co-elute
X-Terra RP_{18} 3.5µm	20mM KH_2PO_4 + Acetonitrile (80:20)	Isocratic	0.5	10	25	214 - 400	Benzamide, R_t = 2.118; Benzoic Acid, R_t = 3.790; Benzonitrile, R_t = 5.164	Benzamide, 224.1; Benzoic Acid, 229.9; Benzonitrile, 222.13	Benzoic Acid and Benzonitrile co-elute

7.2 Separation and Detection of β-Amino Nitriles and their Related Products Formed During Enzymatic Biotransformation Using Reverse Phase High Performance Liquid Chromatography

BACKGROUND

Rhodococcal bacteria have enzymes that can be used to afford biotransformation of nitrile-containing substrates. In an effort to produce chemical compounds using "green procedures", these enzymes can be utilised to accomplish these biotransformation. Various β-amino nitriles were evaluated to determine the ability of the enzymes to produce the hydrolysis products.

PRINCIPLE OF THE METHOD

The method was developed to separate the nitriles used from the products formed. The typical transformation would involve the transformation of nitrile group to the amide and thereafter to the corresponding acid. Retention and separation was achieved on a hydrophobic reversed-phase C-18 HPLC column. Detection was accomplished using a PDA (photodiode array) UV (ultra-violet) detector.

SAFETY

The risks associated with the chemicals and the reagents to be handled are summarised below:

Acetonitrile : Toxic, flammable

Trifluoroacetic acid : Harmful, corrosive

Samples and standards should be tightly closed and stored in a freezer at -20ºC.

Flammable reagents must be kept away from any source of ignition.

Laboratory coat, safety glasses and safety gloves must be worn when handling these chemicals and samples.

All samples and standard solutions to be disposed of in accordance with good laboratory practise.

Instrumentation must be operated according to good laboratory practise and the operating procedures recommended by the manufacturer.

REAGENTS

Acetonitrile : HPLC grade. 99.9% pure is suitable
Trifluoroacetic acid : Chemically pure, 99.0% pure is suitable
Ultra-pure water : 18 Ωm is suitable
Membrane filters : 0.45 µm nylon membrane

Preparation of Mobile Phase:
Prepare 0.1% (v/v) trifluoroacetic acid (TFA) solution by pipetting 1 ml TFA into 1 l volumetric flask (A grade) and diluting with ultrapure water. Filter the solution through a 45 µm nylon membrane and degas in ultrasonic bath for 10 min. Acetonitrile is used as supplied by the manufacturer.

APPARATUS

Liquid chromatography : Waters 2690 separation module coupled with Waters Diode Array Detector 996 or equivalent
Data handling : Waters Empower 2 software or equivalent
Analytical Column : Waters X-Terra MS18 3.5 µm, 3.0 × 50 mm (i.d × L) column

PROCEDURE

Set up the Instrument:

HPLC Instrument : Waters 2690 separation module coupled with Waters Diode Array Detector 996
Column : Waters X-Terra MS18 3.5 µm, 3.0 × 50 mm (i.d. x L) column
Eluent : A – 0.1 Trifluoroacetric acid
B – Acetonitrile. Composition is variable
Flow rate : 0.3 $ml.min^{-1}$ to 0.5 $ml.min^{-1}$

Pump mode : Isocratic
UV Detection wavelength : 210 nm - 400 nm
Run Time : 15 - 20 min.
Injection Volume : 10.0 µl
Column Temperature : 25ºC
Note: The chromatographic system has to be optimised for at least 1 h in advance with a column flow rate of 0.5 ml.min^{-1} before the injection of samples.

Data Manipulation
All data manipulation is done by using the Empower 2 Software. During typical run, the PDA data channels are monitored and stored. The correlation coefficient of the calibration graph must not be less than 0.99.

Injection of standards and samples
Inject the blank twice at the beginning of a sequence and inject the check standard three times, after every ten injections of samples, inject check standard once throughout the sequence and at the end of the sequence inject the check standard three times.

RECOVERIES:
The average recoveries for each of the compounds should be between 95 -105 with the %RSD of less than 5%.

7.3 Enantiomeric Separation of β-Amino Nitriles

SAMPLE PREPARATION

Samples are prepared prior to submission for analysis by the initiator in an appropriate solvent, thus they are injected neat.

REAGENTS AND MATERIALS

n- Hexane: Labscan, 95% HPLC Grade

Propan-2-ol: Labscan, HPLC Grade

Membrane filters: 45 µm nylon membrane

Note: Filter all solvents through 45 µm nylon membrane and degassed in ultrasonic bath for 10 min.

PROCEDURE

Set up the Instrument:

Liquid Chomatograph System: Waters 600-MS Separation Module equipped with Waters 717 Autosampler, Waters 2486 UV/Visible Detector, and Empower 2 Chromatography Management data software

Column	: Chiralpack® AD-H, 250 × 4.6 mm, 5 µm; Chiralcel® OD-H, 250 × 4.6 mm, 5 µm.
Eluents	: A -n-Hexane (HPLC Grade) B – Isopropanol (HPLC Grade)
Flow rate	: 1 $ml.min^{-1}$
Pump mode	: Isocratic
Detection wavelength	: 210 nm
Run Time	: 30 min.
Injection Volume:	10.0 µl
Column Temperature	: 25ºC

Note: The chromatographic system has to be optimised for at least 1 h in advance with a column flow rate of 1 $ml.min^{-1}$ before the injection of samples.

Calculation of the results

The correlation coefficient of the calibration graph must not be less than 0.99. The calculations are performed automatically using the Empower 2 Software.

7.4 Enantiomeric Separation of β-Amino Amides using Crownpak CR(+) Column

SAMPLE PREPARATION

Samples are prepared prior to submission for analysis by the initiator in an appropriate solvent, thus they are injected neat.

REAGENTS AND MATERIALS

Water : Nano-pure water (18.2 Ω Ohm).

Membrane filters : 45 µm nylon membrane

Perchloric Acid : Merck, 70%

Preparation of Perchloric Acid : Accurately weigh 16.3 g (70% Perchloric acid) and dilute to 1000 ml with nano-pure water (pH 1). Measure 100ml of 70% Perchloric acid (pH 1), dilute to 1,000ml with nano-pure water (pH 2.00), filter through 0.45µm or 0.22µm nylon membrane and degassed in ultrasonic bath for 10 min.

PROCEDURE

Set up the Instrument:

Liquid Chomatograph System: Waters 2690 Separation Module equipped with Photodiode Array Detector 996, and Empower 2 Chromatography Management data software

Column : Crownpak CR (+), 150 × 4 mm

Eluent : 16.3 $g.l^{-1}$ Perchloric Acid, pH 2in Nano-pure water

Flow rate : 0.25 $ml.min^{-1}$

Detection wavelength : 210nm, 220 nm or 225 nm

Run Time : 20 – 60 min.

Injection Volume : 10.0 µl

Column Temperature : 10ºC

Note: The chromatographic system has to be optimised for at least 1 h in advance with a column flow rate of 0.25 $ml.min^{-1}$ before the injection of samples.

Calculation of the results

The correlation coefficient of the calibration graph must not be less than 0.99. The calculations are performed automatically using the Empower 2 Software.